



COLLAPSE OF *ZOSTERA NOLTII* SEAGRASS BEDS EFFECTS ON NEMATODE COMMUNITY STRUCTURE IN THE MIRA ESTUARY (SOUTHWEST COAST OF PORTUGAL): ANALYSIS OF ESTUARINE NEMATODES ASSEMBLAGES IN EARLY RECOVERY

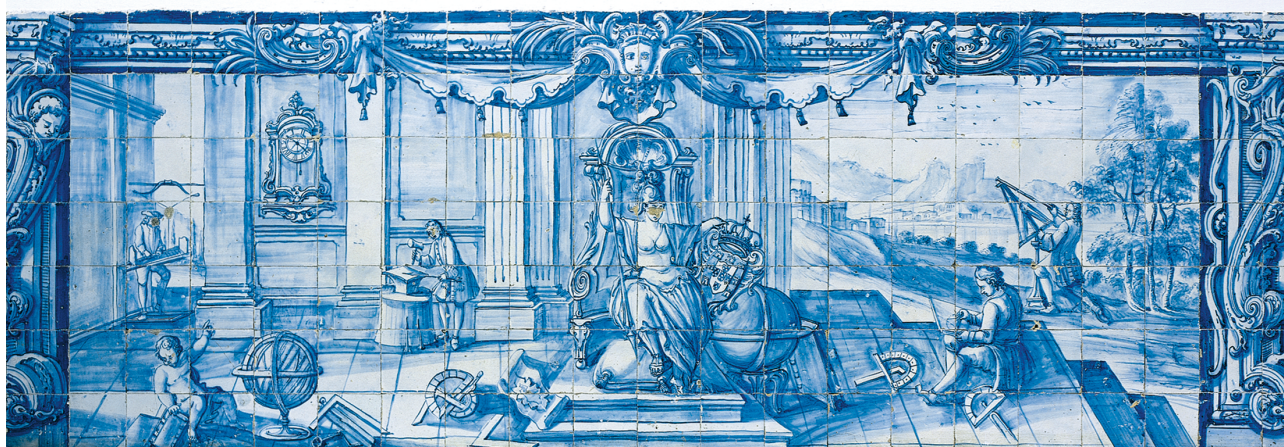
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‘It's very important to transcend places that hold us.’

Rubin ‘Hurricane’ Carter

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Abstract

This research focuses on the benthic nematode assemblages response during a natural recovery of the habitat, after a major collapse of the *Zostera noltii*, in Mira estuary (SW coast, Portugal). The main aim was attained by comparing nematode assemblages distribution patterns of density, diversity, trophic composition, biomass and morphometric attributes, between the stable ecological condition of the seagrass habitat and the early recovery process. During the early recovery no evident temporal patterns of the assemblages were observed, and a high density and diversity was registered. However, in comparison to the stable ecological condition, during the early recovery, the nematodes density decreased, while the diversity, biomass and morphometric attributes increased. The dual stable isotopes allowed to determine that carbon inputs associated with seagrass beds extend well beyond the vegetation boundaries. The essence of ecological functioning was maintained after the habitat loss, being possible to predict that a “good ecological state” can be achieved.

Efeito do colapso da planta marinha *Zostera noltii* na estrutura de uma comunidade de nemátodes no Estuário do Mira (Sudoeste de Portugal): Análise de Nemátodes Marinhos no Início do Processo de Recuperação

Este trabalho centra-se na resposta das comunidades de nemátodes bentónicos durante a recuperação natural do habitat, após um grande colapso de *Zostera noltii*, no estuário do Mira (Costa Sudoeste de Portugal). O principal objetivo foi alcançado através da comparação de padrões de distribuição das comunidades de nemátodes em termos de densidade, diversidade, composição trófica, biomassa e atributos morfométricos, entre a condição ecológica estável da pradaria marinha e o processo de recuperação inicial. Durante o início da recuperação não foram observados padrões temporais evidentes das comunidades e foi observada uma alta densidade e diversidade. No entanto, comparando com o estado ecológico estável, durante o início da recuperação, a densidade de nemátodes diminuiu, enquanto a diversidade, biomassa e atributos morfométricos aumentaram. Os isótopos estáveis permitiram determinar que as adições de carbono associados às pradarias marinhas estendem-se bem além dos limites da vegetação. A essência do funcionamento ecológico foi mantida após a perda do habitat sendo por isso possível prever que possa ser alcançado um "bom estado ecológico".

General Introduction

Estuary: definition and general features

The term ‘estuary’, with its origin in the 16th century, is derived from the Latin word ‘aestuarium’, itself derived from ‘aestus’ that means tide (Elliott & McLusky 2002; Hardisty 2007). The first definition of estuary accepted by most scientists was provided in 1967 (Pritchard 1967). According to Pritchard, an estuary could be defined as ‘a semi-enclosed coastal body of water, which has a free connection with the open sea, and within which sea water is measurably diluted with fresh water derived from land drainage’. In 1980, a new estuary definition consisted on ‘an inlet of the sea reaching into a river valley as far as the upper limit of tidal rise, usually being divisible into three sectors: a) a marine or lower estuary, in free connection with the open sea; b) a middle estuary subject to strong salt and freshwater mixing; and c) an upper or fluvial estuary, characterized by freshwater but subject to strong tidal action. The limits between these sectors are variable and subject to constant changes in the river discharges’ (Fairbridge 1980). Fairbridge considered that the upper limit of the estuary is the upstream limit of tidal penetration whereas Pritchard considered that it is the upstream limit of salt penetration. This new definition added tidal freshwater areas to the estuary.

These definitions however do not explicitly mention the existence of tides, they are basically based on a salinity distinction and do not seem to take into account estuaries located in the southern hemisphere. While in northern hemisphere most rivers flow all year and estuaries remain open to tidal influence at all times, in the more southern estuaries, there is an extended period of dry weather in the summer (Elliott & McLusky 2002). In this case, the inflowing fresh water cease for many months and tidal connection with the open sea may be lost. As a consequence, salinity may increase in

summer due to evaporation and decrease significantly in winter. This intermittent feature of estuaries was not included in Pritchard's and Fairbridge's definitions but it is in Day's (1989) definition: 'an estuarine system is a coastal indentation that has a restricted connection to the ocean and remains open at least intermittently. The estuarine system can be subdivided into three regions: a) a tidal river zone, a fluvial zone characterized by lack of ocean salinity but subject to tidal rise and fall of sea level; b) a mixing zone (the estuary proper) characterized by water mass mixing and by the existence of strong gradients of physical, chemical, and biotic quantities reaching from the tidal river zone to the seaward location of a river mouth bar or ebb-tidal delta; c) a nearshore turbid zone in the open ocean between the mixing zone and the seaward edge of the tidal plume at full ebb tide.' However Day's definition lacks the mention of the salinity regime. In 2010, Potter et al. studied the effects of water loss due to evaporation on salinity and proposed the definition: 'an estuary is a partially enclosed coastal body of water that is either permanently or periodically open to the sea and which receives at least periodic discharge from a river(s), and thus, while its salinity is typically less than that of natural sea water and varies temporally and along its length, it can become hypersaline in regions when evaporative water loss is high and freshwater and tidal inputs are negligible' (Potter et al. 2010). This high range of salinity has consequences in terms of the variety of species present. The estuarine ecosystem will not only include species that cannot tolerate wide fluctuations in salinity (stenohaline) and species that tolerate wide ranges of salinity (euryhaline) but will also include species that tolerate high salinity situations. These last were recently included in the Remane diagram, which describes species diversity and dominance according to their salinity tolerance, until a salinity value of 60 (Whitfield et al. 2012). However hypersalinity does not seem to be included in the recent definition by Whitfield and Elliot (2012): 'An estuary is a

semi-enclosed coastal body of water which is connected to the sea either permanently or periodically, has a salinity that is different from that of the adjacent open ocean due to freshwater inputs, and includes a characteristic biota’.

The European Union Water Framework Directive (2000/60/EC, European Union) considered that estuaries are included in the term ‘Transitional Waters’ defined as ‘bodies of surface water in the vicinity of river mouths which are partially saline in character as a result of their proximity to coastal waters but which are substantially influenced by freshwater flow’. This definition is less restrictive and encompasses tidal estuaries and non-tidal brackish water lagoons (Elliott & Whitfield 2011; McLusky & Elliott 2007). This term is widely used by several countries, although not always as defined by the EU Water Framework Directive (McLusky & Elliott 2007). There are still needed further discussions to reach a detailed and widely appropriate definition.

Estuaries are among the most productive and complex ecosystems, providing different habitats such as shallow open waters, river deltas, tidal flats, saltmarshes and seagrass beds offering favorable conditions for a variety of flora and fauna such as invertebrates, fish and birds (McLusky & Elliott 2004). They are also among the most valuable ecosystems, due to the wide range of goods and services they provided such as tourism, sediment and nutrient cycling, protection against floods, etc (Costanza et al. 2007).

One of the most important features of estuaries is the salinity gradient, ranging from a low salinity upstream to euhaline downstream areas, or even hypersaline areas. Salinity is the main responsible for the distribution of estuarine species in the waters (Whitfield 1999). Water circulation due to tidal and freshwater currents transports sediments and organisms and is responsible for nutrient and oxygen cycling (Gibson 2003). Estuaries are one of the most important paths for the transfer of sediments between land and sea or ocean. Sediment sizes vary from less than 2 μm to over 4 mm. Fine sediments, such

as clay and silt, frequently called mud, dominate in most estuaries. The deposition of the particles along the estuary depends on the currents, on the particle size and composition and on cohesion. When currents are very strong most particles are transported, however, when waters start to slack, the heavier particles, such as sands, gravel and cobbles begin to deposit while fine particles remain in suspension. The deposition of mud is mostly dependent on cohesion, a physical property that consists on the ability of fine sediments to bind together, aggregate or flocculate, resulting in an increase of their weight and consequent deposition (Uncles et al. 2006). This process occurs especially in the upper part of the estuary where lower salinity regimes are found (Biaty et al. 2010). Estuary substrate is rich in organic matter and due to bacteria associated in decomposition that consume oxygen, conditions few centimeters below the surface become anoxic.

Estuaries are increasingly being influenced by the effects of anthropogenic activities contributing to their deterioration (Dauvin & Ruellet 2009). These include the removal of native vegetation from the catchments of some estuaries resulting in habitat destruction; the stock impoverishment due to overfishing or bivalve harvesting (Figure 1); the disturbance on the sediment water interface that increase stress levels and affect local animal communities; the decrease on water quality due to effluent discharges from agriculture or industries and nutrient enrichment (Cloern 2001; Austen et al. 2002). Large inputs of nutrients is the main reason for eutrophication in estuaries. This problem increases in estuaries where the tidal water movement is limited and therefore has less ability to wash out the overload nutrients to the sea. Effects of eutrophication include the development of opportunistic macroalgae and reduction of seagrass beds, development of anoxia and hypoxia events, mortality of fish and invertebrates (Cloern 2001).



Figure 1. Bivalve harvesting in Mira estuary (Photo: P. Materatski, 2010)

One is to note that estuaries are highly variable habitats and naturally stressed ecosystems. This natural stress is not always distinguished from anthropogenic stresses but is of major relevance to determine the Ecological Quality Status for transitional waters (Elliott & Quintino 2007). The human activities, together with the effects of climate changes, require that managers get a good understanding of the physico-chemical characteristics of estuaries so that they can develop measures for preventing their further degradation and for maintaining their biodiversity. The Driver-Pressure-State-Impact-Response (DPSIR) is a framework that has been introduced in order to evaluate human and ecological impacts in estuarine and coastal ecosystems and that together with the existing legislation for the protection of water resources such as the EU Water Framework Directive (2000/60/EC) and the Marine Strategy Framework

Directive (2008/56/EC) and other directives worldwide, will contribute for an integrated management of estuarine ecosystems (Borja & Dauer 2008).

Seagrasses: definition, world distribution and losses

Seagrasses are flowering plants, or angiosperms, that developed several adaptations that allow them to live their full lifecycle submersed in marine environments. Such adaptations include internal gas transport, epidermal chloroplasts and marine pollination and dispersal (Robertson 1984; Orth et al. 2006). Seagrasses have evolved from a single lineage of monocotyledonous flowering plants about 100 million years ago. They have a very low taxonomic diversity, comprising ca. 0.02 % of the total angiosperm species, in contrast to other marine species such as salt marsh plants, mangroves and marine algae which have evolved from multiple evolutionary lineages and are highly diverse (Orth et al. 2006; Short et al. 2007). They maintained their ability to produce flowers, fruits and seeds (Ackerman 2006). Seagrass species can reproduce asexually, through horizontal rhizome growth that becomes physiologically independent but genetically identical to the mother plant, or sexually, through the seeds (Kuo & Kirkman 1987). Seeds of most seagrass species are poorly adapted for dispersal, however some species can form banks of seeds which is a major survival advantage for plants when subjected to disturbed environments. Despite their low diversity, seagrass beds deeply influence the environments in coastal waters, and are frequently called as ‘ecological engineers’ due to their important role in structuring pelagic and benthic assemblages (Bos et al. 2007). They are economically extremely important, providing high-value ecosystem services when compared with other marine and terrestrial habitats (Costanza et al. 1997). They are among the world’s most productive coastal ecosystems (Duarte & Chiscano 1999). They provide food for marine herbivores and serve as nursery and refuge habitat for

several species of invertebrates and fish with high economical impact (Beck et al. 2001; Heck & Valentine 2006). Their proximity to other habitats such as salt marshes, mangroves or coral reefs promotes trophic transfers and a cross-habitat utilization by these animals contributing to their abundance (Beck et al. 2001; Valentine & Heck 2005). Seagrass stimulates nutrient cycling, produces large quantities of organic carbon, stabilizes water flow and promotes sedimentation, contributing to the minimization of coastal erosion and to the filtration of nutrient inputs to the coastal ocean (Hemminga & Duarte 2000; Heiss et al. 2000; Boström et al. 2006). Moreover, seagrasses are considered as biological sentinels, or ‘coastal canaries’ (Orth et al. 2006) mostly because they are highly sensitive to environmental deterioration and widespread geographical distribution. They require high levels of light to provide oxygen to their roots and rhizomes, due to their large amounts of non photosynthetic tissue (Terrados et al. 1999), making them highly sensitive to variations in environmental changes involving water clarity. Changes in seagrass distribution are reflected on benthic assemblages densities, species composition and trophic composition and may influence spatial and temporal patterns distribution (Boström & Bonsdorff 1997; Boström et al. 2006).

There are currently about 60 species of seagrasses belonging to five different families: *Hydrocharitaceae*, *Cymodoceaceae*, *Posidoniaceae*, *Zosteraceae* and *Ruppiales* distributed in coastal areas across the world (Green & Short 2003; Duarte & Gattuso 2010). In contrast to other coastal habitats such as salt marshes, mangroves and corals, seagrasses can be found either in tropical and in temperate regions. In the north Atlantic temperate region there is a low seagrass diversity, dominated by *Zostera* and *Ruppia* species, being *Zostera marina* the main species. In the north Pacific temperate region there is a high seagrass diversity dominated by the temperate species of *Zostera* and

Phyllospadix genera. The Mediterranean region has diverse temperate and tropical seagrass flora, dominated by *Cymodocea*, *Zostera* and *Posidonia* species, being *P. oceanica* the prevalent species. The temperate southern coastlines of Australia, Africa and South America show a low to high diversity of temperate seagrasses, dominated by *Posidonia*, *Zostera*, *Amphibolis* and *Halophila* species. The tropical Atlantic and tropical Indo-Pacific regions have high diversity of seagrasses including *Cymodocea*, *Enhalus*, *Halodule*, *Halophila*, *Syringodium*, *Thalassia* and *Thalassodendron*, being the most dominant species *Thalassia testudinum* (Green & Short 2003; Short et al. 2007). Among *Zostera* species, *Zostera noltii* Hornem is distributed along the coasts of the Atlantic ocean, from the south of Norway to the south of the Mauritanian coast (den Hartog 1970; Cunha & Araújo 2009). It is also present in the Mediterranean, Black, Baltic, Caspian and Aral Seas and in Canary islands (Diekmann et al. 2010; Short et al. 2007). The Portuguese coast is dominated by three seagrass species: *Cymodocea nodosa*, *Zostera marina* and *Zostera noltii*. These were found in 18 different portuguese estuaries, being *Z. noltii* the most widely distributed (15,74 km²) appearing in 10 of these sites (Cunha et al. 2013).

Seagrasses are being threatened all over the world (Hughes et al. 2009). The abundance and distribution of seagrasses are a response to a wide variety of natural stresses but most important of all, to human pressures that degrade water quality (Orth et al. 2006). Seagrass populations have been decreasing since 1879, when the first records were made. Between 1879 and 2006, the measured area of seagrass loss was 3370 km², and currently the total area of seagrass is estimated as being of ca. 177000 km² (Waycott et al. 2009). Loss rates varied from 0.9% per year prior to 1940, to 7% per year since 1990, clearly showing an increase in the last decades. In the 1930's, the 'wasting disease' caused by a marine slime mold like, *Labyrinthula zosterae* (Rasmussen 1977;

Muehlstein 1989), destroyed over 90% of all eelgrass (*Zostera marina*) along North Atlantic coast of America and Europe. There are several other direct or more indirect threats that seagrasses are subjected to, such as climate changes, which are responsible for the increase of sea level and temperature; alterations in water quality; increased loading of nutrient and sediments; introduction of non-native species, among others (Waycott et al. 2009). Seagrasses are vulnerable to any conditions that reduce light, such as eutrophication and turbid conditions caused by land disturbance. Seagrasses constitute areas often used as subsistence fisheries due to their easily accessible locations and therefore susceptible to overexploitation. They are also subjected to destruction due to urbanization of coastal zones or other direct human impacts. In particular, *Z. noltii* has a very thin rhizome (0.5-2 mm) which unables it to grow vertically, making this species susceptible to burial. *Z. noltii* biomass varies during the year, reaching higher values when temperatures and light are high (Adão 2003). This seagrass is typically found on the interface between marine and terrestrial environments, in the intertidal zone (Moore & Short 2006) where it is particularly vulnerable to climate changes and anthropogenic stresses (Valle et al. 2014). In addition, the red alga *A. armata* and *Caulerpa racemosa* are also a threat to these populations (Cabaço & Santos 2007; Cunha et al. 2013).

The Mira estuary is located in southwest of Portugal. In the 1980-90's, when the first studies were made, seagrass (*Z. noltii* and *Z. marina*) covered an area of ca. 0.8 km² (Andrade 1986; Cunha et al. 2013) (Figure 2A). After a flood event, in 2008, *Z. noltii* disappeared completely in the Mira estuary, leaving a muddy area full of dead rhizomes (Adão personal communication) (Figure 2B). This estuary is included in a protected area subjected to low human stresses (Adao et al. 2009) and the causes of such a seagrass loss are not yet clear. Change in sedimentation dynamics is one possible

hypothesis. These are known as one of the most common causes of seagrass loss (Orth et al. 2006) and have been observed in Mira estuary in the last decade (Adão personal communication). In 2009 the seagrass bed in Mira estuary start presenting signs of natural recovery consisting in small patches with an irregular spatial and temporal distribution (Cunha et al. 2013) (Figure 2C). The most recent studies, dated from 2009, show that *Z. marina* was very rare and *Z. noltii* covered an area of ca. 0.075 km² (Cunha et al. 2013). At present, these values may be lower as since 2009 there were several periods that no *Zostera* species were observed (Adão personal communication) (Figure 2D).

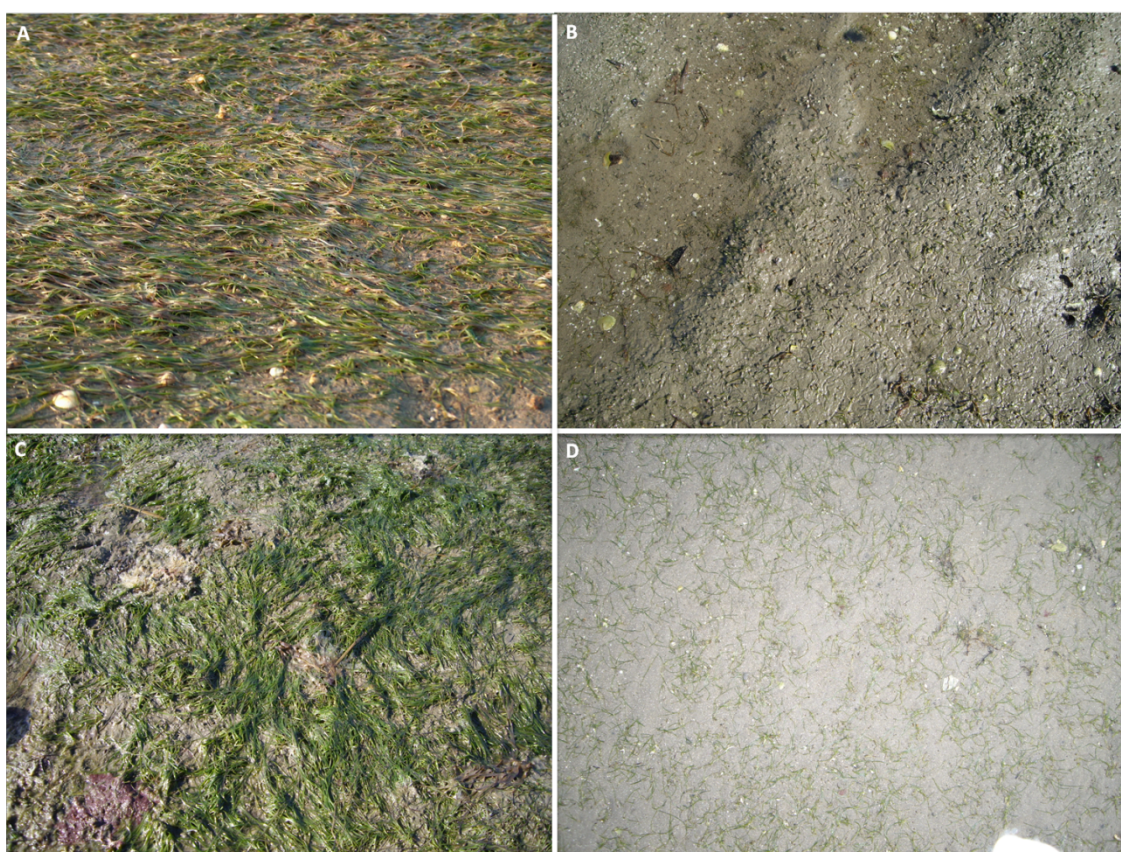


Figure 2. Zostera noltii presence in Mira estuary: A- before *Z. noltii* disappearance (1995); B – after *Z. noltii* disappearance (2009); C – dense patch of *Z. noltii* in early recovery process (2010); D – sparse *Z. noltii* in early recovery process (2011). (Photos: H. Adão and P. Materatski)

Despite of all the threats *Z. noltii* is subjected all over the world, this species is currently listed as least concern on the International Union for the Conservation of Nature (IUCN) Red List of Threatened Species. In fact, impacts to seagrasses in general have received limited public attention (Waycott et al. 2009). However, disturbances that affect seagrasses, not only have impact on seagrass itself but also have impacts in the flora and fauna communities associated with the seagrass, leading to changes in estuarine food webs, species population biomass, abundance and diversity, and representing high economic losses. The loss of seagrass has also a great influence in the physical environment such as the water quality, erosion, specially of fine particles, and sediment resuspension (Baeta et al. 2011; Grilo et al. 2012). Several measures promoting favourable growing conditions have been implemented in recent decades in several places. Seagrass re-establishment through transplantation has been attempted worldwide with different levels of success (Fonseca et al. 1998; Green & Short 2003). In Tampa Bay, Florida, the effort to reduce nutrient inputs over the past 2 decades have resulted in a recovery of a seagrass area of 27 km². In Mondego estuary, Portugal, after a severe period of eutrophication, the alteration of estuarine hydraulics and control of fishery, increased the seagrass area in over 1.5 km² from 1997 to 2002 (Cardoso et al. 2005).

The numerous complex changes that occur due to seagrass loss make the natural recovery of seagrass beds often a very slow process, that can take from decades to centuries. For this reason, efficient monitoring and management strategies together with public awareness, are of great importance to prevent and reverse the loss of seagrass beds (Ganassin & Gibbs 2008; Grilo et al. 2012).

Benthic fauna: definition and meiofauna features

‘Benthos’ comes from a Greek word that means ‘depth of the sea’. The benthic community comprehends the organisms that live on (epifauna) or in (infauna) the bottom of a water body. It includes a highly diverse range of organisms from bacteria to plants (phytobenthos) and animals (zoobenthos). The classification of benthic organisms is generally made according to the organisms size. Macrobenthos includes the larger organisms, over 0.5 mm in size (e.g. bivalves, crustaceans and gastropods). Meiobenthos includes organisms less than 0.5 mm in size but are retained on a 39 μm mesh size sieve (e.g. nematodes) and microbenthos comprises organisms smaller than 39 μm (e.g. bacteria, flagellates and diatoms). Exact dimensions vary among researchers.

Meiobenthos are highly diverse. They can occur in both freshwater and marine habitats, from shallow waters to deep sea. They can be found living in all kinds of sediments from mud to coarse gravel, as well as in rooted vegetation, moss, macroalgae, sea ice and animal structures (Higgins & Thiel 1988). Meiofauna abundance values frequently range from 10^5 to 10^7 ind/ m^2 with 10^5 to 20^5 ind / m^2 in estuaries and shallow coastal environments. Biomass generally ranges from 1 to 2 g C/ m^2 in shallow waters with estuarine mud flats presenting the highest values (Coull 1988). The abundance, diversity and distribution of meiofaunal organisms depend mainly on the sediment particle size, temperature and salinity. However, they are also influenced by other factors such as food resources, oxygen, turbidity, hydrodynamic regime (Coull 1999; Shabdin 2006). All these factors make these organisms to be heterogeneously spatially distributed. A large scale (m to km) variability has been suggested as due to changes in physical factors, such as those involving sediments and small scale (cm to m) variability due to biological interactions (Findlay 1981). Some macrobenthos organisms are, during their

juvenile stages, part of the meiobenthos, called temporary meiofauna. Permanent meiofauna, i.e. species that are meiobenthic throughout their life cycle, include, among others, members of the phyla Mystacocarida, Rotifera, Nematoda, Polychaeta, Copepoda, Ostracoda and Turbellaria (Higgins & Thiel 1988). Within estuaries, meiofauna, namely nematodes have a very important role, they support several higher trophic levels and they also facilitate the process of biomineralization. In addition, these organisms have a high sensitivity to stresses especially due to their small sizes and low mobility, which together with their high diversity, short generation times and ubiquitous distribution, make them excellent bio indicators for monitoring environmental changes (Coull 1999; Kennedy & Jacoby 1999).

Nematodes: definition, classification, reproduction and trophic relations

Nematodes are usually the most abundant group in most marine sediments, comprising 60 to 90% of the total meiofauna with the highest values observed in sediments sized < 330 μm (Coull 1999; Shabdin 2006). They can reach densities of up to several million individuals per m^2 , that, although low, corresponds to a higher total carbon input than any other meiofauna (Moens & Vincx 1997). Nematodes are also highly diverse, especially in muddy sediments (Heip et al. 1985). Among other factors, nematode success in estuarine sediments is due to 1) their burrowing capacity that, in combination with their small sizes allow them to occupy the interstitial spaces in sediments; 2) their tolerance to environmental stresses; and 3) their diversification in buccal structures that enable them to exploit a broad range of food (Bouwman 1983).

Phylum Nematoda is divided into two classes: Secernentea and Adenophorea. About 4000 of the 20000 nematode species are free-living marine forms and from these only 2, both belonging to genus *Rhabditis*, are from the Class Secernentea. Most nematodes

belonging to Class Adenophorea present caudal glands, bristles and conspicuous amphids as opposition to members of Class Secernentea (Riemann 1989). Most marine nematodes vary in size from 1 to 3 mm and most nematodes are dioecious, i.e. have separate male and female individuals with males being usually smaller than females. Reproduction is usually by copulation. Some species are ovoviviparous, embryos develop inside eggs within the mother's body before they hatch. From the egg to adult nematodes suffer developmental changes and pass through four juvenile stages that are sometimes impossible to identify morphologically in terms of species (Warwick 1981). Nematodes have a relatively long fertile period causing an overlap of generations (Woombs & Laybourn-Parry 1986).

Nematodes are highly diverse with respect to size, shape and type of food. Based on that, Wieser (1953) classified nematodes in four trophic groups according to the size of their buccal cavity structure and to the presence of teeth: 1A – selective deposit feeders and 1B – non-selective deposit feeders (both without teeth, and with small and large buccal cavities, respectively); 2A – epistrate feeders and 2B – omnivores or predators (with small teeth and powerful mandibles, respectively) (Figure 3).



Figure 3. Four genera of nematodes belonging to four feeding types: A - Selective deposit feeder, *Terschellingia* sp.; B- Non-selective deposit feeder, *Camacolaimus* sp.; C- Epistrate feeder, *Euchromadora* sp.; D-Predator/omnivor, *Sphaerolaimus* sp. (Photos: P. Materatski)

Moens and Vincx (1997) proposed modifications to Wieser classification based on observations of the feeding behaviours of living nematodes in estuary, establishing six trophic groups: 1) microvores, that feed exclusively on bacteria; 2) ciliate feeders, that feed mostly on ciliates but also on bacteria; 3) deposit feeders, that feed on bacteria, diatoms and other microalgae; 4) epigrowth feeders, that feed on diatoms and other microalgae; 5) facultative predators, that feed on several items, including detritus and other nematodes; and 6) predators, that feed mainly on nematodes. The abundance of each trophic group varies according to site and environmental conditions. Nematodes are opportunistic feeders and that means they may change their feeding strategy as an adaptation to available food.

One way to study food web trophic relations is by using stable isotopes (Peterson & Fry 1987). Isotopes are forms of an element with the same number of protons and electrons but with a different atomic mass due to number of neutrons (Fry 2006). Stable isotopes of carbon and nitrogen ratios have been commonly used in marine ecosystems (Lepoint et al. 2004; Baeta et al. 2009; Carlier et al. 2009; Lebreton et al. 2011; Ouisse et al. 2012). Naturally occurring carbon and nitrogen stable isotope characteristics of consumers reflect the isotopic characteristics of their diet and the nutrient source at the base of the food web (Fry & Sherr 1984; Fry 2006). Ratio of carbon isotopes ($\delta^{13}\text{C}$) of consumers and resources are usually very similar and can be used to evaluate the ultimate sources of carbon for an organism when the isotopic signature of the sources are different. The nitrogen isotope ratios ($\delta^{15}\text{N}$) of consumer tissues (a measure of a substance's isotope ratio relative to atmospheric nitrogen) is influenced by trophic interactions and also by the basal food web isotopic composition (Vander Zanden & Rasmussen 1999; Post 2002). The $\delta^{15}\text{N}$ of a consumer is typically enriched by 3 to 4 ‰ relative to its diet, showing a measurable offset between different trophic levels (Vander Zanden & Rasmussen 2001; Post 2002) and allowing the estimation of the trophic position. Dual stable isotopes may therefore be used to study both resources and trophic position of consumers (Moens et al. 2005). The use of stable isotopes however requires a minimum of biomass, which in the case of very small animals, such as nematodes, usually means the use of hundreds of individuals (Moens et al. 2005). Because of that, dual stable isotopes of nematodes has been reported for nematodes on a community level (Riera et al. 1996; Riera et al. 1999; Riera & Hubas 2003), which may lead to inaccuracy since nematode species have ecological different behaviours (Moens et al. 2005). To date, studies concerning nematode at species level are scarce, Carman and

Fry (2002) and Moens et al. (2005) are the few concerning nematode diet studies using dual stable isotopes and none of these were made on seagrass habitats.

Studying trophic interactions within nematode communities can help in achieving a better understanding on the importance of these communities on benthic systems and contribute to a better knowledge of the ecosystem functioning.

Nematodes as bioindicators, their abundance and distribution

The use of nematodes as indicators of the environmental conditions has been supported due to their variety of characteristics that make them representative of overall ecosystem status (Coull 1999). Nematodes are ubiquitous, they occur in any environment, polluted or not. They are usually in greater density and diversity than other members of meiofauna which makes them suitable for the detection of different types and levels of stress. They have short generation times and sublethal effects of pollutants on reproduction, growth rates, longevity and behaviour can be determined in days. They are smaller and easier to sample when compared to macrofauna. They are typically relatively sedentary, do not rapidly migrate from stressful conditions and respond rapidly to various environmental gradients from macro to microscale ranges (Coull & Chandler 1992).

Among the most important environmental factors that affect nematodes, namely their distribution, density and diversity, are: the size of the sediment particle, salinity and temperature (Coull 1999). Other factors such as oxygen and food availability, turbidity, hydrodynamic regime, topography, seagrass distribution, as well as anthropogenic pressures, may also explain nematodes spatial (vertical and horizontal) and temporal distribution (Heip et al. 1985; Fleeger & Decho 1987).

Seasonal variations in nematode abundance and diversity have been mostly attributed to the influence of temperature, but also to seasonal rainfalls, food abundance, anoxic depth levels, bioturbation and disturbance. Nematode densities are usually higher in the spring/summer when temperatures are higher (Tietjen 1969) and are lower at low temperatures mostly because of the increase of generation times of estuarine nematode species at lower temperatures as well as because of the decrease on the rates of nutrient assimilation (Heip et al. 1985; Moens & Vincx 2000). There are few studies that show higher densities in winter seasons however in these studies, it is stressed that the seagrass canopy may have an important effect in food supply (Alongi 1987). Nematode communities from temperate and sub-tropical habitats have shown changes in their feeding type from season to season (Alongi 1990). Several studies have shown that the annual pattern of total abundance and species composition is repeatable from year to year and that annual patterns vary within sites in an estuary (Coull 1999; Fisher 2003). Spatial distribution of nematodes is mostly heterogeneous and is influenced by factors at a scale from centimetres to metres (e.g. food availability) to a scale from metres to kilometres (e.g. variation in physical gradients) (Hall et al. 1994; Montagna 1991). Horizontal distribution depends mainly on sediment composition, salinity and temperature. The density of marine nematodes is higher in fine sediments and lower in coarse sediments, however in terms of diversity it is the opposite, it tends to exist a higher diversity in coarse sediments than in fine sediments (Heip et al. 1985). In addition, nematodes species in coarse sediments tend to be more robust, which is probably an adaptation to unstable coarse sediments (Warwick 1971; Heip et al. 1985). Remane's model shows the effect of a salinity gradient on the density of benthic invertebrate species in the Baltic Sea. It shows that densities are higher in marine waters, moderate in fresh water and lower in water with salinity between 5 and 8. To

include estuarine conditions, in 2002, Attrill related the species density with the variation in salinity over time, rather than with average salinity, and found that species diversity was lower where salinity was most variable and higher in stable marine and freshwaters (Attrill 2002; Ferrero et al. 2008). Nematode densities have shown to present the same behaviour (McArthur et al. 2000). The diversity of nematodes has also shown to be influenced by latitude. A higher diversity was observed in temperate areas than in equatorial or polar regions (Boucher 1990; Lamshead et al. 2000; Mokievsky & Azovsky 2002). However, salinity and sediment characteristics on the scale of metres to kilometres, proved to be more important in explaining community structure than latitudinal differences on the scale hundreds of kilometres (Soetaert et al. 1995). Subtidal, intertidal and supratidal zones have different physicochemical properties and therefore influence nematodes distribution and abundance (Hourston et al. 2005). Changes in tidal amplitude and current velocity change the distribution and accumulation of sediments and consequently the meiofauna communities (Smol et al. 1994). Nematodes are the most abundant meiobenthic organisms in intertidal zones. Nematode densities have shown to be lower in water depths greater than 200 m than in shallow waters however these differences have been attributed to other variables such as food availability and sediment characteristics (Grémare et al. 2002; Lamshead 2003; Udalov et al. 2005).

On a vertical scale of centimetres, near de surface, factors that affect horizontal distribution have an as important role as in vertical distribution. Nematodes vertical distribution depends on a variety of biological, physical and chemical factors: penetration of oxygen, water content, sediments, temperature, biogenic structures such as seagrass roots, food sources, proximity to surface (Escaravage et al. 1989; Giere 1993; Steyaert et al. 1999). Meiofauna has mostly been found in the 2 cm near the

surface depending mainly on the type of sediment and oxygen (Smith & Coull 1987; Vincx 1996), factors that are related. The finer the sediments, the more near the surface the nematodes are and that seems to happen because in fine sediments, such as silt and clay, the oxygen decreases more rapidly with depth. Due to the fact that most meiofaunal organisms need oxygen, they are limited by the Redox Discontinuity Layer (RDL) which divides aerobic and anaerobic sediments (Coull 1999). Besides the type of sediment, oxygen level is also affected by water pumping caused by macroinvertebrates and wave action and therefore these variables may also affect vertical distribution of benthic species (Cullen 1973). Changes in vertical distribution of nematodes have also been shown in relation to tidal cycles (Steyaert et al. 2001). Certain nematode species migrate towards the sediment surface during inundation of tidal flat and returned when the tidal was low. Vertical separation may reduce predatory interactions which explains the high number of species found in a restricted patch (Joint et al. 1982).

Several assessment tools based on diversity (Margalef Index, d ; Shannon-Wiener diversity, H') or on ecological strategies (Index of Trophic Diversity and Maturity Index), are used to highlight the distributions of nematode communities and their responses to environmental changes. The two ecological indicators based on diversity (Margalef Index, d ; Shannon-Wiener diversity, H') can indicate loss of biodiversity and also suggest a reduction in functional biodiversity when presenting low values, especially in stressed environments that are subjected to organic enrichment, human disturbance and physical stressors (Mirto & Danovaro 2004; Frascchetti et al. 2006; Bianchelli et al. 2008; Danovaro et al. 2008; Gambi et al. 2008). However, in natural stressed environments a higher biodiversity may mean the adaptation or ability of benthic systems to perform the key biological and biogeochemical processes that are crucial for their sustainable functioning (Danovaro et al. 2008). The Index of Trophic

Diversity (ITD) is based on the density contribution of each feeding-type as classified by Wieser (1953) (Heip et al. 1985). ITD ranges from 0.25 (highest trophic diversity, i.e., each of the four trophic guilds accounts for 25% of the nematode density), to 1.0 (lowest trophic diversity, i.e., one trophic guild accounts for 100% of the nematode density). Recently it has been used the reciprocal value of the trophic index (θ^{-1}), so that the highest values of the index correspond to the highest trophic diversity. The ITD is generally used to correlate the trophic diversity of nematodes with pollution levels (Heip et al. 1985; Mirto et al. 2002). Changes in ITD are usually only highlighted when strong variations in the assemblage structure occur, which means that its use as the only tool in monitoring programs may sometimes be questionable (Vincx & Heip 1987). The general principle of the Maturity Index (MI) is based on the different strategies of nematode assemblages in relation to different disturbances (Bongers 1990; Bongers et al. 1991). The MI assigned to nematode genera on the *c-p scale*, ranges from 1 (colonizers) to 5 (persisters), where taxa with rapid growth and reproduction and usually high tolerance to disturbance are considered colonizers, whereas slow-growing and more sensitive taxa which thrive well in fairly stable and pristine environments are considered persisters (Bongers 1990; Bongers et al. 1991). Thus, the *c-p* scores reflect life-history characteristics associated with r- and K- selection for colonizers and persisters, respectively (Bongers & Bongers 1998; Bongers & Ferris 1999). As happens with the ITD, the use of the MI as the only tool in monitoring programs is questionable, since it can sometimes only distinguish the extreme conditions of disturbance (Moreno et al. 2011).

The classical nematode community analysis in terms of density, diversity, genera composition and functional diversity is well documented (Castel et al. 1989; Guerrini et al. 1998; Ndaro & Olafsson 1999; Fisher & Sheaves 2003; Fonseca et al. 2011; Alves et

al. 2013). More limited information exists in terms of nematodes morphometry (length, width and length/width ratio) and biomass. Nematodes show a wide range of different sizes and body proportions that result from environmental adaptations. These indicators reflect specific modes of life in terms of feeding strategies, life history and diversity may therefore be used to study nematodes ecosystems (Warwick & Price 1979; Vidakovic & Bogut 2004; Moens et al. 2007; Leduc et al. 2010; Quang et al. 2014). The study of biomass and allometric attributes for monitoring changes, in comparison with taxonomic identification, is easier, not requiring high taxonomic skills, less time-consuming and has a lower cost, with obvious implications in environmental management (Vanaverbeke et al. 2003). Total biomass may be estimated from numerical abundance, and mean individual biomass can be measured based on length and width measurements. The Andrassy's formula has been widely used for biomass calculations, $W=(L \times D^2)/(1.6 \times 10^6)$ where W is the mass (μg wet weight), L is the length (μm) and D is the body diameter (μm) (Andrassy 1956). A ratio of 0.25 may then be assumed to convert wet weight into dry weight (Heip et al. 1985). The length/width (L/W) ratio is a measure of nematodes body shape. According to this ratio, nematodes have been classified into categories. Nematodes with a low L/W ratio < 6 were classified as short or stout and nematodes with a L/W ratio > 14 were classified as long/slender (Ratsimbazafy et al. 1994; Soetaert et al. 2002). In 2007, Schatzberger et al. classified as stout, nematodes with a low L/W ratio < 18 ; as slender, nematodes with a L/W ratio of 18-72 and as long/thin, nematodes with a high L/W ratio > 72 (Schratzberger et al. 2007). Slender and long nematodes move fast through the sediment and are more representative of the feeding type of predators/omnivores and epistrate feeders whereas stout nematodes have a more reduced mobility and are more representative of the non selective and selective deposit feeders groups, having a more

opportunistic behavior (Warwick 1971; Soetaert et al. 2002; Quang et al. 2014). Other studies comparing nematodes body size and trophic groups have been done, showing however different results, suggesting that there is not such a linear relation and that other factors may be involved (Tita et al. 1999). Longer nematodes have high generation times and high maturity indexes. They invest more in growth than in reproduction (Bongers 1999; Ferris & Bongers 2006; Vanaverbeke et al. 2007). Nematode lengths and consequently L/W ratios are affected by several factors such as dissolved solids in water and nitrate concentrations in sediments; sediment particle size, chlorophyll a and total pigment concentration and oxygen concentrations. High nematode length and high L/W ratio (longer and thinner nematodes) have shown a positive correlation with: total dissolved solids in the water and nitrate and nitrite concentrations in the sediment as well as coarser sediment particles; and a negative correlation with oxygen concentrations and chlorophyll a and total pigment concentration. The decrease of chlorophyll a and total pigment concentrations may be explained by the decrease on the number of deposit feeders (and therefore decrease of average lengths) that mainly feed on detritus and microalgae (Warwick 1971; Heip et al. 1985; Soetaert et al. 2009; Losi et al. 2013; Quang et al. 2014). Nematode widths have shown to be affected by coliform concentrations, nitrite concentrations in sediment and dissolved oxygen. Nematode widths show a positive correlation with coliform concentrations and negative correlation with nitrite concentrations and dissolved oxygen (Atkinson 1973). Individual and total biomass are negatively correlated with dissolved oxygen. Nematodes with a higher dry weight require lower oxygen consumptions, suggesting that nematodes are well adapted to conditions of low oxygen. Total biomass has shown a negative correlation with coarser sediments, increasing when the percentage of sand decreases and when percentage of silt increases in opposition to

individual biomass that increases with the increase of coarser sediments (Quang et al. 2014). This can be explained due to the higher density of nematodes found in finer sediments. Other studies however have shown higher individual biomass in muddy sediments than in sandy sediments (Tita et al. 1999). These differences may be explained because many other sediment characteristics other than grain size, such as organic content, water content, redox potential, porewater oxygen concentrations, among others, may affect nematodes body size (Fleeger et al. 2011).

Anthropogenic pressures that affect nematodes

Nematodes abundance and distribution may also be affected due to anthropogenic pressures. Several studies have focused on the effects of global warming, organic enrichment, hydrocarbon spills and other contaminants such as copper, lead, zinc, iron and cadmium. Since the industrial revolution carbon dioxide (CO₂) and other greenhouse gases (GHG) have been increasing, mostly due to the use of fossil fuels, with a direct effect in global warming. Among global warming consequences are the increase of the frequency and magnitude of extreme weather events, ocean acidification, rise of sea level, increase of air temperature. In estuaries, the most important impacts are associated with flood or drought events with important effects in nematodes (Attrill & Power 2000). Man's activities have resulted in high inputs of nutrients and organic matter creating an unbalanced ecosystem (Austen & Warwick 1995). Nematodes increase in abundance along a gradient of increasing organic enrichment, until a point where conditions deteriorate so much that nematodes are absent. Although nematode assemblages from muddy estuaries are not affected with low inputs, high inputs result in a decrease of species diversity (Schratzberger & Warwick 1998). This knowledge may have management implications for the marine environment in that if the same amount of

organic matter is administrated in low inputs it has a lower effect on nematode communities. The high concentrations of polycyclic aromatic hydrocarbons (PAHs) in aquatic environments are usually associated with discharges of petroleum and its derivatives, due to shipping and coastal activities such as land-based industrial inputs and domestic discharges (Burns & Saliot 1986; Louati et al. 2001). PAHs concentrate in sediments and pass to benthos through direct contact or ingestion, that subsequently pass them to their predators (Marshall & Coull 1996; DiPinto & Coull 1997). Impacts of pollutants on benthic communities have been studied (Lee et al. 1981; Coull & Chandler 1992). Nematodes diversity has shown to decrease in presence of such pollutants however, in terms of abundances, there have been shown different results. Different dosages and toxicity of pollutants, sedimentary conditions, different species susceptibility and the fact that benthic communities from more contaminated areas are more tolerant to pollutants may help to explain the different abundance variations (Di Toro et al. 1991; Millward et al. 2004).

Response of nematodes to anthropogenic pressures such as disappearance or decrease of some species may significantly influence interactions among other benthic taxa (Carman et al. 1997). It may lead to food limitation for animals that have nematodes as their obligatory food source and, on the other hand, nematode food sources such as microalgae may increase as a consequence of reduced feeding. All these changes in the food web may have serious implications for the functioning of the whole marine ecosystem (Attrill & Power 2000).

Nematode communities on seagrass beds of *Zostera*

Several studies show that vegetated sediments support higher abundances, biomass and diversity of infauna in general than surrounding unvegetated sediments (Edgar et al. 1994; Boström & Bonsdorff 1997; Webster et al. 1998; Hemminga & Duarte 2000).

The vegetation reduces water movement and increases sedimentation of fine particles thus altering particle size structure and the availability of food (Castel et al. 1989). It allows the creation of complex habitats and substrates for several organisms, offering them shelter from predation, as well as feeding and nursery areas (Neckles et al. 1993; Boström et al. 2006; Fredriksen et al. 2010). In nematodes communities some studies demonstrated highest abundances and diversity in seagrass beds (Escaravage et al. 1989; Alongi 1987; Fisher & Sheaves 2003), though other results obtained did not show that evidence (Castel et al. 1989; Danovaro 1996; Leduc & Probert 2011; Fonseca et al. 2011) suggesting that more complex interactions may be involved.

More studies are needed to understand which and how nematode species respond to the complex environment created by seagrasses. The recovery processes focused on biological interactions, are an important baseline to an emerging field in aquatic ecology such as “Restoration Ecology” (Verdonschot et al. 2012). It is however difficult to assess where an ecosystem is positioned along a trajectory of recovery (Latimer et al. 2003) and when a coastal ecosystem can be declared recovered (Elliott et al. 2007). The increase of the ecosystem quality by structural and functional “natural recovery” implies a passive ongoing process, and may not result in a return to the original state but instead, in a newly created ecosystem regaining quality (Elliott et al. 2007).

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General Aims

The disappearance and early recovery of the *Z. noltii* seagrass beds of the Mira estuary created an interesting ‘natural experiment’ allowing to examine the fundamental ecology of nematode assemblages during passive natural recovery. Nematode assemblages are generally influenced by complex physical and biological processes that surround them. Therefore, the early and passive natural recovery process of the seagrass beds of *Z. noltii* in the Mira estuary, may influence the variation in nematode distribution patterns in terms of density, diversity, trophic composition as well as biomass and morphometric attributes. The identification of temporal and spatial patterns in nematode communities is therefore an essential step towards the understanding of the processes that structure the communities.

The main aims of this study are:

Chapter I - Investigate the temporal and spatial variability patterns of density, taxonomic and functional diversity as well as composition of the nematode assemblages associated with the early natural recovery process of the seagrass beds of *Z. noltii* in the Mira estuary.

Will the new environmental conditions of the early recovery process, with sparsely distributed and small sized seagrass patches, increase the temporal and spatial variability patterns of density, taxonomic and functional diversity as well as the composition of the nematode assemblages?

The temporal and spatial variability of the nematode assemblages was assessed at two sampling sites, with three stations each, on five sampling occasions. The following null

hypotheses were tested: *i*) there would be no differences at different temporal sampling events of the nematode assemblage density, species and trophic composition; *ii*) there would be no differences in spatial variability patterns of the nematode assemblage density, species and trophic composition during the early recovery of *Z. noltii*.

Chapter II - Investigate changes in patterns of nematode assemblage composition and biodiversity, trophic composition and life strategies between different environmental conditions of the seagrass habitat: "Before" the habitat loss in stable condition, and "After", during the early recovery of seagrass beds.

Will the nematode assemblage in the new environmental conditions "after" the collapse of seagrass beds *versus* the stable condition "before" the habitat loss, increase the temporal and spatial variability patterns of density composition and biodiversity, trophic composition and life strategies at different sampling occasions and sites after the collapse?

The following null hypotheses were tested: *i*) There would be no differences in nematode assemblage composition and biodiversity, density and trophic composition during both environmental conditions, "before" and "after"; and *ii*) there would be no differences in nematode assemblage composition, density and trophic composition at different sampling occasions during both environmental conditions.

Chapter III – Investigate the nematodes morphometric descriptors, length, width, L/W ratio and biomass, as complementary information to the classical structural analysis of nematode assemblages (Materatski et al. in prep., Chapter II), between different environmental conditions of the seagrass habitat: "Before" the habitat loss in stable condition, and "After", during the early recovery of seagrass beds.

Will the nematode assemblage in the new environmental conditions "after" the collapse of seagrass beds *versus* the stable condition "before" the habitat loss, increase nematode biomass, length, width and L/W ratio at different sampling occasions and sites?

The two distinct environmental conditions, before and after *Zostera* disappearance, temporal and spatial distribution were analyzed using two sampling sites and five sampling occasions. The following null hypotheses were tested: *i*) there would be no differences on nematodes length, width, L/W and biomass at different sampling events, before and after *Zostera* disappearance; *ii*) there would be no differences on nematodes length, width, L/W and biomass at different temporal (sampling occasions) and spatial (site A and B) samplings.

Appendix I - Document food web structure and elucidate the contribution of potential carbon sources to macrofauna diets in an estuarine seagrass habitat, using stable carbon and nitrogen isotopes. We address the following research questions: (1) Do seagrass-associated sources contribute substantially to the diet of macrobenthos? If so, we would expect differences in resource utilization in the seagrass bed vs adjacent unvegetated sediments. (2) Is there temporal variation in resource utilization by macrofauna?

Appendix II - Assess the principal carbon resources of the nematode and harpacticoid copepod assemblages, at the species, genus and family levels, in *Z. noltii* seagrass beds and in adjacent bare sediments. In addition, examine the validity of existing mouth-morphology based nematode feeding guilds, based on their trophic position and resource utilization as revealed by the stable isotope data obtained in this study. If current guild classifications represent real functional groupings, then resource utilization and trophic level within feeding guilds should be very similar, while it would

differ between guilds. We hypothesized that microphytobenthos (MPB) would be the principal carbon resource for the majority of taxa in bare sediments. In vegetated sediments, seagrass- associated resources (i.e. seagrass detritus and epiphytes) could also contribute, and higher sedimentation rates would likely raise the contribution of suspended particulate organic matter (SPOM) to meiofauna diets.

Thesis outline and publications

The thesis is divided into four parts, General Introduction, Body of the thesis, General Conclusion and Appendices. The body of the thesis is itself divided into three chapters. Each chapter consists in an scientific paper in the form as will be submitted for publication and is an autonomous part. The Appendices I and II of this thesis are an integrant part of this research, consisting of two scientific papers already published in collaboration with the University of Gent. These papers focused on the trophic dynamics of the macrofauna, meiofauna and benthic nematodes during the early recovery of *Z. noltii* and the results obtained were determinant to understand the ecosystem functioning and to help in the interpretation of the results obtained by analysis of the nematode assemblages abundance and diversity. These papers have already been included in a Master Thesis and are therefore included in this thesis as appendices.

In the general discussion, there are made considerations deduced from the main results of the different chapters as well as from the main results of Appendices I and II.

Chapter I:

Materatski, P.; Vafeiadou, A.-M.; Moens, T; Adão, H. (2014). Benthic nematode assemblage composition and diversity during a natural recovery process of *Zostera noltii* seagrass beds.

Chapter II:

Materatski, P.; Vafeiadou, A.-M.; Ribeiro, R; Moens, T; Adão, H. (2014). A comparative analysis of benthic nematodes assemblages before habitat loss and during

the early recovery of *Zostera noltii* seagrass beds in Mira estuary (Southwest Coast of Portugal).

Chapter III:

Materatski, P.; Ribeiro, R; Adão, H. (2014). Biomass and morphometric attributes of nematodes in Mira estuary (Southwest Portugal) before *Zostera noltii* disappearance and during early recovery process.

APPENDICES I and II:

Vafeiadou, A.-M.; **Materatski, P.**; Adão, H.; De Troch, M.; Moens, T. (2013). Food sources of macrobenthos in an estuarine seagrass habitat (*Zostera noltii*) as revealed by dual stable isotope signatures. *Marine Biology*, 160: 2517-2523.

Vafeiadou, A.-M.; **Materatski, P.**; Adão, H.; De Troch, M.; Moens, T. (2014). Resource utilization and trophic position of nematodes and harpacticoid copepods in and adjacent to *Zostera noltii* beds. *Biogeosciences*, 11: 4001–4014.

Results from these thesis have been orally presented in several conferences:

Materatski, P., Vafeiadou, A.M., Moens, T., Adão H. (2014). Benthic nematode assemblage response during the early phase of a natural recovery process of seagrass beds *Zostera noltii*. 54th ECSA, 12-16 May, Sesimbra.

Vafeiadou, A.-M., **P. Materatski**, H. Adão, M. de Troch, T. Moens, T. (2013). Food web analysis of meiobenthos in estuarine seagrass bed. Proceedings of the FiftIMCo 22-26 July South Korea.

Materatski, P. and Adão, H. (2013). Response of Nematode Assemblages After a Major Collapse in Seagrass Beds of *Zostera noltii*. Simpósio de Ciências Marinhas, 17-18th June, CO-Oceanography Center, Campus da FCUL, Lisboa.

Adão, H., **Materatski, P.**, Vafeiadou, A.M., Moens, T. (2012). Benthic Nematodes Assemblages as Indicators of Spatial Heterogeneity in Early Natural Recovery of *Zostera noltii* Seagrass Beds. 2nd International Symposium on Nematodes as Environmental Bioindicators, 5-6 July. Gent.

Materatski, P., Vafeiadou, A.M., Moens, T., Adão H. (2012). Recovery of Benthic Nematodes Assemblages after a Major Collapse in Seagrass Beds of *Zostera noltii*. 50th ECSA, 3-7 June, Venice.

Materatski, P., Vafeiadou, A.M., Moens, Adão H. (2011). Recovery of benthic nematode assemblages after a major collapse of *Zostera noltii* seagrass. World Conference on Marine Biodiversity (WCMB), 26-30 September, Aberdeen, Scotland, (UK) p.37

Chapter I

Benthic nematode assemblage composition and diversity during a natural recovery process of *Zostera noltii* seagrass beds

Abstract

Recently there has been a growing interest in the recovery trajectories of the coastal ecosystems. The stable seagrass beds of the Mira estuary located in a natural park, disappeared completely, however they have begun presenting slight symptoms of passive natural recovery, characterized by strong heterogeneous distribution. This study was designed to investigate the spatial and temporal variability patterns of densities, species composition and trophic composition of the benthic nematode assemblages associated with this early recovery process, at two sampling sites, three stations each, and at five sampling occasions. The environmental variables measured give an indication of similar ecological conditions and patterns at both sites. The nematode densities and the number of species were generally high and the genera composition comparable to several intertidal muddy sediments. The most important spatial pattern emerged from the nematode density distribution of both sampling sites, while at sampling stations level a low horizontal variability was registered. The temporal patterns of the nematode density, trophic composition and diversity were not evident. The functional responses of the nematode assemblages revealing ability to withstand to natural variability imposed during the early recovery process and predicted the good ecological functioning of this ecosystem can be achieved.

Keywords: Nematodes, *Zostera noltii*, natural recovery, biodiversity, spatial and temporal distribution.

Introduction

Nematodes are the most diverse and numerically dominant metazoans in aquatic habitats, with a wide distribution varying from pristine to extremely polluted habitats. They are widely regarded as ideal organisms to study the potential ecological effects of natural and anthropogenic disturbances in aquatic ecosystems due to their ubiquitous distribution, high abundance, presence across the food web, intimate association with sediments, fast reproduction and rapid life histories (Schratzberger et al. 2000; Austen & Widdicombe 2006; Alves et al. 2013). Furthermore, investigations have highlighted the importance of the link between nematode diversity and ecosystem functioning (Danovaro et al. 2008). These attributes give nematodes strong advantages over other potential indicators, as they can reflect changes in environmental conditions over spatial and temporal scales, making them more informative in the assessment of estuarine and marine biological integrity (Norling et al. 2007; Danovaro et al. 2008; Patrício et al. 2012).

Seagrass beds comprise some of the most heterogeneous landscape structures of shallow-water estuarine/marine ecosystems in the world. They are declining worldwide (Hughes et al. 2009). These beds have important ecological roles in coastal ecosystems and provide high-value ecosystem services when compared to other marine and terrestrial habitats (Costanza et al. 1997). They are typically considered as ‘ecosystem engineers’ due to the role they play in structuring pelagic and benthic assemblages (Bos et al. 2007). They are highly productive, influence the structural complexity of the habitats, stabilize water flow and promote sedimentation, and often enhance biodiversity (Orth et al. 2006; Boström et al. 2006). The presence and density of seagrass vegetation is reflected in benthos densities, species composition and trophic

composition, and may influence its spatial and temporal patterns (Boström & Bonsdorff 1997; Boström et al. 2006). Many studies have reported that seagrass beds harbour higher biomass, abundance, diversity and productivity of benthic organisms than unvegetated sediments (Edgar et al. 1994; Boström & Bonsdorff 1997; Webster et al. 1998; Hemminga & Duarte 2000; Hirst & Attrill 2008). Their high sensitivity to environmental deterioration and widespread geographical distribution also make seagrasses useful “miner’s canaries” of coastal deterioration (Marbà et al. 2006; Orth et al. 2006).

There have been numerous reports of seagrass decline worldwide indicating that seagrass habitats are undergoing a global crisis with important consequences for coastal biodiversity, environmental status and economy (Boström et al. 2006; Hughes et al. 2009). Unprecedented decline of *Zostera* sp. meadows has also been reported in Portuguese estuaries in the last decade (Cunha et al. 2013). For instance, in 2008 the *Zostera noltii* Hornem. beds of the Mira estuary disappeared completely, leaving behind a bare muddy area (Adão personal communication; Cunha et al. 2013). This estuary, together with the Mira River, is included in a protected area and is considered relatively undisturbed and subjected to only slight human-induced pressures (Costa et al. 2001; Adao et al. 2009). The causes of the seagrass loss have not yet been determined, but there is speculation that important changes in sedimentation dynamics have resulted in large-scale alteration of seagrass habitat and are thus potentially the major driver of seagrass habitat loss (Fourqurean & Rutten 2004).

During 2009 the *Z. noltii* bed of Mira estuary began presenting slight symptoms of natural recovery, characterized by pulses with a spatial and temporal irregularly distribution of the small-sized seagrass patches, which change in habitat configuration. Therefore, the distribution of seagrasses has become strongly heterogeneous, both

spatially and temporally (Adão personal communication; Cunha et al. 2013). The disappearance and early recovery of the *Zostera noltii* beds in the Mira estuary create an interesting ‘natural experiment’ allowing to examine the fundamental ecology of nematode assemblages during the early natural recovery processes of seagrass beds. Benthic organisms are generally influenced by complex and interacting physical and biological processes, leading to variation in their distribution patterns (Schratzberger et al. 2008). The identification of temporal patterns in the benthic community structure is therefore an essential step towards understanding the processes that structure ecological communities (Underwood & Chapman 1996; Gallucci et al. 2009). Moreover, understanding the distribution patterns and their interaction with changing environmental conditions is an important baseline for ecological investigations of habitat recovery (Borja et al. 2010; Verdonshot et al. 2012).

Many studies in temperate and subtropical regions have focused on the nematode communities associated with seagrass beds (Tietjen 1969; Alongi 1987; Castel et al. 1989; Ansari & Parulekar 1993; Guerrini et al. 1998; Ndaro & Olafsson 1999; Paula et al. 2001; Somerfield et al. 2002; Fisher & Sheaves 2003; Gambi et al. 2009; Fonseca et al. 2011). Seagrass structural complexity is often an important determinant of the temporal and spatial distribution of benthic nematode assemblages, closely coupled with the physicochemical regime, the trophic dynamics and the biological factors of the environment as competition and predation pressures (Escaravage et al. 1989; Eskin & Coull 1987; Bouvy & Soyer 1989; Ansari & Parulekar 1993; Schizas & Shirley 1996; Ólafsson & Elmegren 1997; Danovaro & Gambi 2002; Adão 2004). Some studies have clearly demonstrated that nematode assemblages have higher abundance and diversity in seagrass beds than in neighbouring bare sediments (Alongi 1987; Escaravage et al.

1989; Fisher & Sheaves 2003), but this is not always the case (Castel et al. 1989; Danovaro 1996; Fonseca et al. 2011).

The main aim of this study was to investigate the temporal and spatial variability patterns of density, taxonomic and functional diversity as well as composition of the nematode assemblages associated with the early and passive natural recovery process of the seagrass beds of *Z. noltii* in the Mira estuary. Our field observations prompted us to include a spatial component, because during the period of our study, no stable seagrass vegetation patches emerged. Instead, low-biomass patches continually emerged, disappeared and re-appeared at slightly different positions, creating a dynamic mosaic of *Zostera noltii* patches interspersed with bare sediment patches. Therefore, we hypothesized that this combined spatio-temporal variability in vegetation cover would also contribute to higher nematode taxonomic and functional diversity of benthic nematode assemblages. The temporal and spatial variability of the nematode assemblages was assessed at two sampling sites, with three stations each, on five sampling occasions.

The following null hypotheses were tested: *i*) there would be no differences at different temporal sampling events of the nematode assemblage density, species and trophic composition; *ii*) there would be no differences in spatial variability patterns of the nematode assemblage density, species and trophic composition during the early recovery of *Z. noltii*.

Materials and methods

Sampling area and design

Sampling was performed in the Mira estuary, south-western coast of Portugal (37°40'N, 8°40'W) (Fig. 1), a small mesotidal system with a semidiurnal tidal regime (amplitude 1-3 m during neap and spring tides, respectively). The estuary has a single channel, 5–10 m deep and up to 400 m wide, which allows tidal influence to extend 40 km upstream. Due to the low, seasonal and limited freshwater input, the lower section of the estuary has a dominant marine signature characterized until 2008 by extensive and homogenous *Z. noltii* meadows, characterized by a strong seasonality with higher biomass in warm months (Cunha et al. 2013). Together with its surrounding area, the Mira estuary is included in a protected area, the Natural Park of 'Sudoeste Alentejano e Costa Vicentina'. This estuary is considered relatively undisturbed and free from major anthropogenic pressures (Costa et al. 2001). The fluctuations of physico-chemical parameters result mainly from natural pressures as: *i*) its morphology, since the terminal section of the river is rather regular and facilitates the upstream tidal penetration, *ii*) a normally reduced outflow determined by the region's annual rainfall distribution, (concentrated between January and March, with the rest of the year being usually dry) (Paula et al. 2006), and *iii*) the dynamic sedimentation. In 2008, *Z. noltii* meadows disappeared completely. Indications of natural recovery have been observed since 2009 (Adão personal communication; Cunha et al. 2013). To evaluate the temporal and spatial distribution patterns of nematode communities during the early recovery of the seagrass, sampling was conducted in the intertidal *Z. noltii* beds at neap low tide, on five sampling occasions: February 2010, June 2010, September 2010, December 2010 and February 2011, at two sites (A, ca. 1.5 km from the mouth of the estuary, and B, 2 km upstream). Samples were collected from three stations (St1, St2, and St3) at each site, with a distance of 50 m between them.

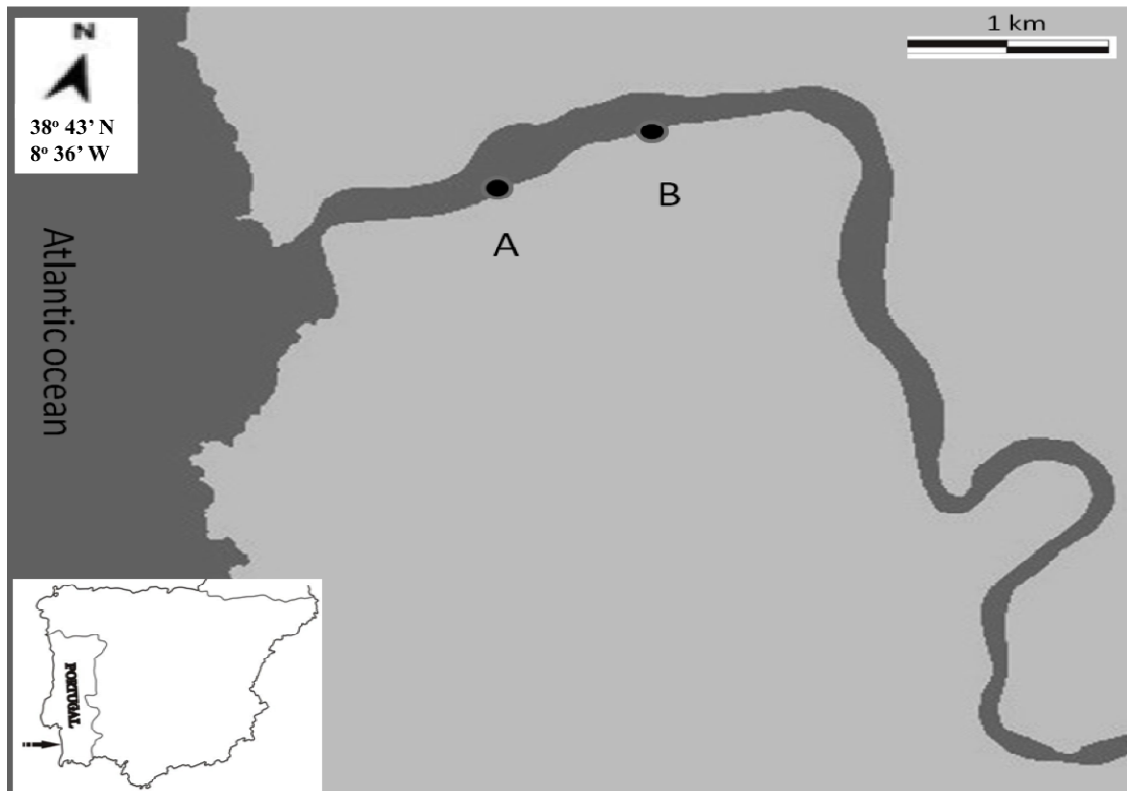


Figure 1. Mira estuary (Portugal): indication of sampling Sites (black circles) - (A, ca., 1.5 km from the mouth of the estuary, and B, 2 km upstream).

Sampling and sample treatment

Biological Data

At each sampling station, three replicate sediment samples of the upper 3 cm were collected using hand corers (4.6 cm inner diameter). All samples were preserved in a 4% buffered formaldehyde solution. Nematodes were extracted from the sediment using a density gradient centrifugation in colloidal silica (Heip et al. 1985). The fixed samples were rinsed on a 1000 μm mesh sieve followed by sieving on a 38 μm mesh. The fraction retained on the 38 μm sieve was washed and centrifuged three times using the colloidal silica polymer LUDOX HS-40 (specific gravity 1.19). The supernatant of each washing cycle was collected again on a 38 μm sieve. After extraction, all nematodes were counted under a stereomicroscope (40 \times magnification). A random set of 120

nematodes was picked from each replicate, transferred through a graded series of glycerol–ethanol solutions, stored in anhydrous glycerol, and mounted on slides (Vincx 1996). Nematodes were identified to genus level using pictorial keys (Platt & Warwick 1988) and online identification keys/literature available in the Nemys database (Vanaverbeke et al. 2014). Nematode genus level is considered a taxonomic level with good resolution to discriminate disturbance effects (Warwick et al. 1998; Moreno et al. 2008; Schratzberger et al. 2008).

Environmental data

Salinity, temperature (°C), pH and dissolved oxygen (DO) (mg L^{-1}) of the overlying water just above the sediment were measured *in situ* using a WTW InoLab Multi 720 field probe. Additionally, at each site and on five sampling occasions, water samples of the overlying water above sediment (small pools) and of the water column were collected for measurement of N and P nutrients ($\mu\text{mol L}^{-1}$) and chlorophyll *a* (mgm^{-3}): nitrate (NO_3^- -N) and nitrite (NO_2^- -N) concentrations were analysed according to standard methods described in Strickland and Parsons (1972) and ammonium (NH_4^+ -N) and phosphate (PO_4^{3-} -P) concentrations were analysed following the Limnologisk Metodik (1992). Chlorophyll *a* (Chl *a*) determinations were performed according to Parsons et al. (1985). At each site and sampling occasion, sediment samples were taken randomly to determine the organic matter content (OM) and grain size. Sediment organic matter content was determined based on the difference between the dry weight of each sample after oven-drying at 60°C for 72 h and the weight obtained after combustion at 450°C for 8 h, and was expressed as a percentage of the total weight. Grain size was analysed by dry mechanical separation through a column of sieves of different mesh sizes, corresponding to the five classes described by Brown &

McLachlan (1990): a) gravel (>2 mm), b) coarse sand (0.500–2.000 mm), c) mean sand (0.250–0.500 mm), d) fine sand (0.063–0.250 mm), and e) silt and clay (<0.063 mm). The relative content of the different grain-size fractions was expressed as a percentage of the total sample weight. *Zostera noltii* was collected randomly on each sampling occasion, three replicate samples were taken at each site (A;B) using sediment hand-corers with a surface area of 141 cm² in area and 30 cm in depth. On each replicate, the roots were separated from the leaves, than were dried in an oven at 60 °C for 48 hours. The leaves and root biomass was estimated by the organic weight and the ash-free dry weight (gm⁻² AFDW—ash free dry weight). Ash-free dry weight was obtained as the weight loss of the dry material after combustion at 450 °C for 8 hours in a muffle furnace (Heraeus KR 170E).

Data Analysis

Univariate and multivariate analyses to detect temporal and spatial changes in the community structure were performed using the PRIMER v6 software package (Clarke & Warwick 2001) with the PERMANOVA add-on package (Anderson et al. 2008).

Environmental variables

A Principal Component Analysis (PCA) of the environmental variables measured was performed to examine patterns in multidimensional data by reducing the number of dimensions, with minimal loss of information. The PCA ordination was based on the average of the environmental factors measured by “Sites” and “Sampling occasions”. Prior to the calculation of the environmental parameter resemblance matrix based on Euclidean distances, data were log (X+1) transformed followed normalization. Selective transformations were required for the water environmental variables, Chlorophyll *a*,

nitrate, nitrite, ammonium and phosphate concentrations, to follow the assumptions for calculating normalized Euclidean distances.

Nematode assemblages

Total density (individuals 10 cm⁻²), genera composition, trophic composition and several ecological indicators, either based on diversity (Margalef Index, d and Shannon-Wiener diversity, H') or on ecological strategies (Index of Trophic Diversity, ITD ; Maturity Index, MI), were calculated using the nematodes dataset, for each site, station and sampling occasion. In order to investigate the trophic composition of the assemblages, nematodes genera were assigned to one of four feeding groups according to Wieser (1953), mainly on the basis of the mouth morphology, including presence or absence of prominent buccal armature. Based on the feeding-type classification from Wieser (1953), the Index of Trophic Diversity (ITD) was calculated (Heip et al. 1985). The reciprocal value of the trophic index (θ^{-1}) was used, so that the higher values of the index correspond to higher trophic diversity.

Nematode genera were assigned a value on a colonizer–persister scale ($c-p$ scale) from 2 (colonizers) to 5 (persisters), where taxa with rapid growth and reproduction and usually high tolerance to disturbance are considered colonizers, whereas persisters are slow-growing and often more sensitive taxa which thrive well in fairly stable and pristine environments (Bongers 1990; Bongers et al. 1991). Thus, the $c-p$ scores reflect life-history characteristics associated with r- and K-selection for colonizers and persisters, respectively (Bongers & Bongers 1998; Bongers & Ferris 1999). The maturity index was then calculated as the weighted average of the individual colonizer–persister ($c-p$) scores as $MI = \sum_{i=1}^n v(i) \times f(i)$ where $v(i)$ is the $c-p$ value of the taxon i and $f(i)$ is the frequency of that taxon.

A two-way permutational analysis of variance (PERMANOVA) was applied to test the null hypothesis that no significant temporal differences (between sampling occasions) and spatial (between sites and stations) existed in the nematode assemblage descriptors: total density, genera composition, d , H' , ITD, and MI. All PERMANOVA analyses were carried out with the following three-factor design: “Sampling occasion”: February 2010, June 2010, September 2010, December 2010 and February 2011 (5 levels, fixed), “Site”: Site A and Site B (2 levels, random), and “Station”: Station 1, Station 2 and Station 3 (3 levels, nested in “Site”).

Nematode density data were square-root transformed in order to scale down densities of highly abundant nematode genera and therefore increase the importance of the less abundant genera in the analysis. The PERMANOVA analysis was conducted on a Bray-Curtis similarity matrix (Clarke & Green 1988). The null hypothesis was rejected at a significance level <0.05 (if the number of permutations was lower than 150, the Monte Carlo permutation p was used). Whenever significant interaction effects were detected, these were examined using *a posteriori* pairwise comparisons, using 9999 permutations under a reduced model. The similarity in communities between sampling occasions, sites and stations were plotted by Principal coordinates analysis (PCO) using the Bray-Curtis similarity measure. The relative contribution of each genus to the average dissimilarities between sampling occasions, sites and stations was calculated using two way-crossed similarity percentage analysis (SIMPER, cut-off percentage: 90%). The relationship between environmental variables at each site and sampling occasion and the structure of the nematode community was explored with the BIOENV procedure (Clarke & Ainsworth 1993), using Spearman’s correlation.

Results

Environmental variables

Based on the environmental variables measured, both sites studied (A and B) were similar on most sampling occasions. As expected, the biomass of *Z. noltii* was very low and registered strong fluctuations throughout the study period.

These variations are clearly demonstrated by the results of the *Z. noltii* biomass that ranged at site A from the lowest values in February 2010 (1.67 g m^{-2}) to the highest values in June 2010 (7.60 g m^{-2}) and September 2010 (7.12 g m^{-2}) shifting to a complete disappearance in December 2010 and re-appearance in February 2011 (1.70 g m^{-2}). Although site B presented slightly higher values in *Z. noltii* biomass, also a strong fluctuation was found. February 2010 showed the highest values (9.50 g m^{-2}) and June 2010 showed a complete absence, with *Z. noltii* re-appearing, with the lowest biomass values, in September 2010 (2.15 g m^{-2}) and December 2010 (2.79 g m^{-2}) reaching the highest values in February 2011 (8.92 g m^{-2}).

Sediment fractions at both sites were dominated by fine sand (0.063-0.250 mm) and mean sand (0.250-0.50 mm), followed by silt and clay (<0.063 mm), coarse sand (0.500–2.000 mm) and gravel (>2 mm). The PCA ordination of the environmental factors showed that the first two components (PC1, 36.0% and PC2, 19.0%) together accounted for about 56% of the variability in the data (Fig. 2). The PCA ordination did not separate site A and B and as expected strongly fluctuations were demonstrated at both sites. The samples from February 2011 at site A were clearly separated from the remaining ones, mainly due to the coarse sediments, presenting higher percentage of gravel, mean sand and also high chlorophyll *a* concentrations. However, a temporal trend was showed in the ordination at both sites, such as December 2010 with high fine sand values, September 2010 with high salinity and June 2010 with high chlorophyll *a*

concentration of the water. February 2010 and 2011 at site B were characterized by the highest organic matter values and February 2010 at site presented the highest nutrient concentration.

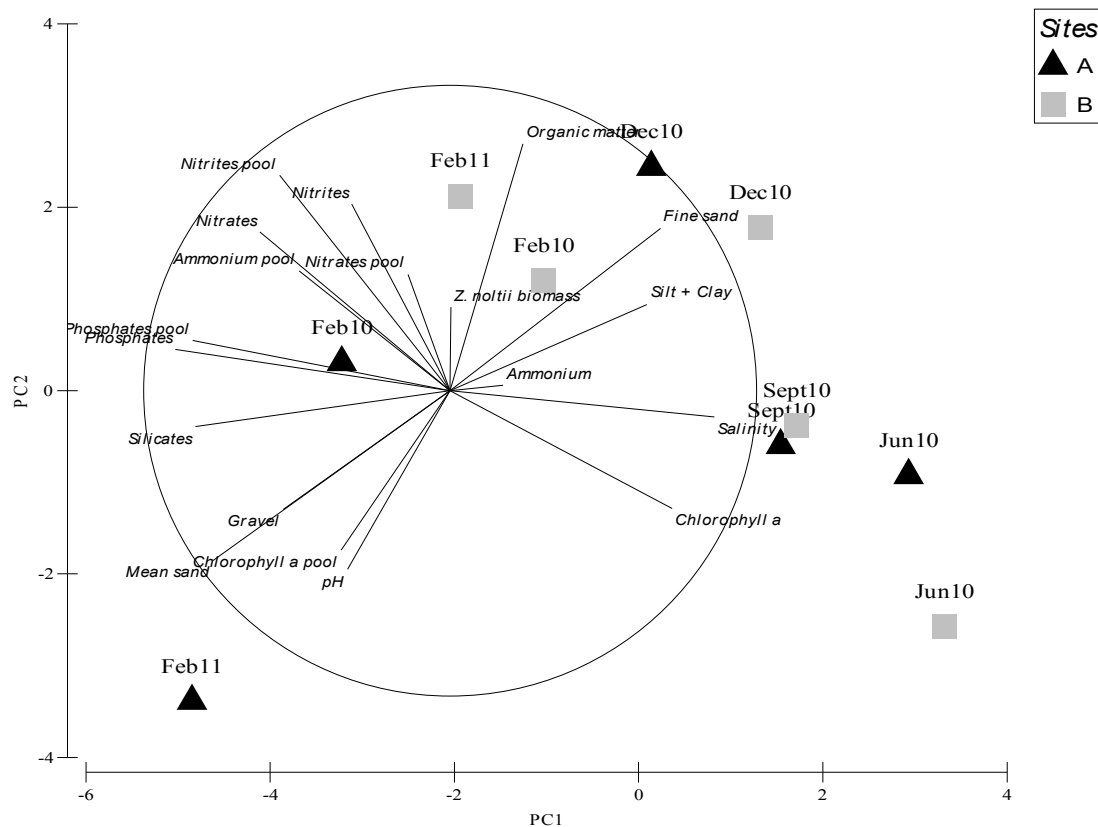


Figure 2. Principal Component Analysis (PCA) plot based on the environmental variables measured at each “Site” A and B and “Sampling occasion” February 2010, June 2010, September 2010, December 2010 and February 2011. PC1 explains 36.0% of the total variance in the data, PC2 19.0%.

Nematode Assemblages - density

Over all sampling occasions the density of nematodes was consistently higher at site B than site A (Fig. 3). Significant differences were obtained between sampling occasions (factor “Sampling occasions”, $p < 0.05$) as well as between sites (factor “Site”, $p < 0.05$). At site B, mean density (\pm SE) was 2611 ± 230 individuals 10 cm^{-2} , with a range

from 1627 ± 287 ind 10 cm^{-2} in February 2011 to 3601 ± 527 ind 10 cm^{-2} in February 2010. At site A, mean density (\pm SE) was 1416 ± 107 ind 10 cm^{-2} with a range from 1221 ± 244 ind 10 cm^{-2} in June 2010 to 1582 ± 188 ind 10 cm^{-2} in September 2010. Moreover, nematode density results showed no significant differences between stations (factor “Stations”, $p > 0.05$). At site B, the lowest density was registered at station 2, 826 ± 169 ind 10 cm^{-2} in February 2011 and the highest at station 3 with 4966 ± 553 ind 10 cm^{-2} in February 2010. At site A, both the lowest and the highest densities were registered at station 1 with 577 ± 168 ind 10 cm^{-2} in June 2010 and 2478 ± 730 ind 10 cm^{-2} in February 2011, respectively.

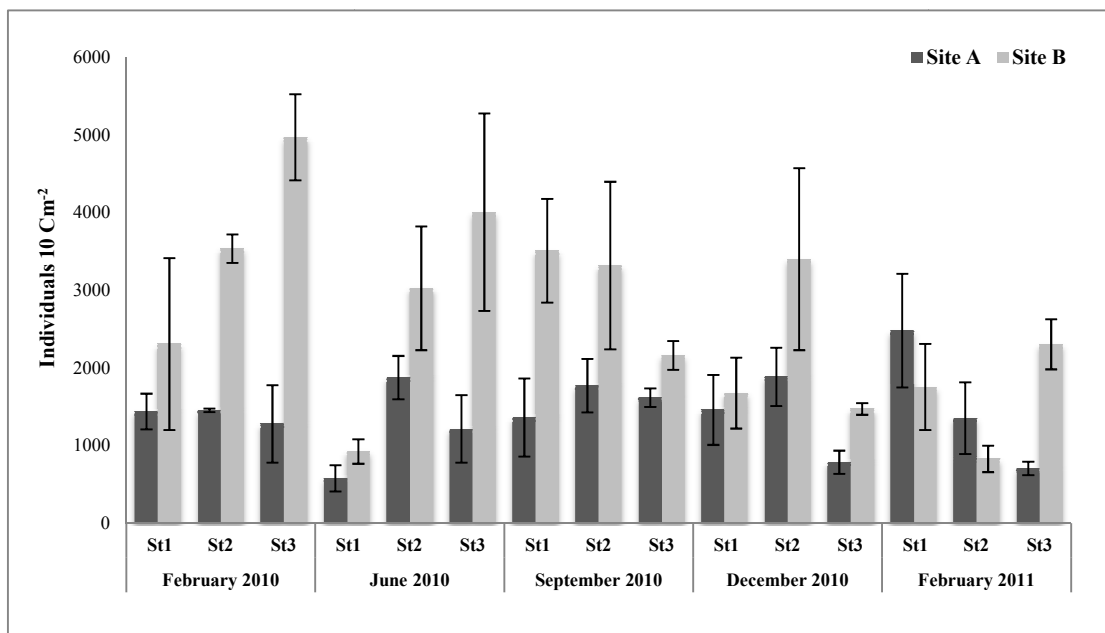


Figure 3. Nematode community (ind 10 cm^{-2}), average density in Stations (St1, St2, St3) and standard error (\pm SE) at Sites (A and B) and Sampling occasions (February 2010, June 2010, September 2010, December 2010 and February 2011).

Nematode Assemblages – structural diversity

At site A, 67 species of nematodes were identified, belonging to 53 genera and 21 families. Most genera belonged to the orders Monhysterida (53.7%), Chromadorida (41.5%) and to a lesser extent Enoplida (4.6%); the dominant families were Linhomoeidae (30.6%), Comesomatidae (19.0%), Desmodoridae (13.4%) and Axonolaimidae (14.0%). The four genera *Terschellingia* (20.7%), *Paracomesea* (17.1%), *Spirinia* (11.4%) and *Odontophora* (10.2%) together comprised nearly 60% of nematode abundances. At site B, we recorded 77 species belonging to 53 genera and 23 families and to the same three orders (Monhysterida (49.7%), Chromadorida (44.8%) and Enoplida (5.5%)). Linhomoeidae (32.4%) and Comesomatidae (24.2%) together accounted for *ca.* 57% of nematode abundances, all other families contributing less than 10%. Among the four most abundant genera, *Spirinia* was replaced by *Linhomoeus* in comparison to site A. These top-4 genera now account for just near half of the total nematode abundances (Table 1).

Species richness and structural diversity based on Margalef Index (d) and Shannon–Wiener values (H') were similar throughout the study period (Fig. 5). The PERMANOVA analysis applied to both indices did not show any significant differences between sites, stations or sampling occasions, nor did it show any significant interaction effect (Table 2).

Nematode assemblages – trophic composition

At both studied sites the nematode assemblages were characterised by non-selective deposit feeders, 1B (mean percentage \pm SE: site A- $34.9 \pm 6.1\%$; site B- $40.2 \pm 7.8\%$) followed by epigrowth feeders, 2A (site A- $32.7 \pm 5.1\%$; site B- $30.2 \pm 6.2\%$), selective deposit feeders, 1A (site A- $22.6 \pm 2.6\%$; site B- $20.0 \pm 2.0\%$) and omnivores/predators, 2B (site A- $9.7 \pm 1.0\%$; site B- $9.3 \pm 1.3\%$). Non-selective deposit feeders (1B) were the most abundant trophic group from September 2010 to February 2011 at both sites,

Table 1. The most abundant nematode genera (individuals 10 cm⁻²), average density in Stations (1, 2 and 3) and standard error (± SE) at Sites (A and B) and Sampling occasions (February 2010, June 2010, September 2010, December 2010 and February 2011), Trophic groups (TG) to each species. Rare genera which contributed with <0.5% of the total density are not included in this table.

| Species | TG | February 2010 | | | | | | June 2010 | | | | | | September 2010 | | | | | | December 2010 | | | | | | February 2011 | | | | | |
|---------------------------|----|----------------|-----|-----|----------------|-----|------|----------------|-----|-----|----------------|-----|-----|----------------|-----|-----|----------------|-----|-----|----------------|-----|-----|----------------|-----|-----|----------------|-----|----|----------------|-----|-----|
| | | Site A Station | | | Site B Station | | | Site A Station | | | Site B Station | | | Site A Station | | | Site B Station | | | Site A Station | | | Site B Station | | | Site A Station | | | Site B Station | | |
| | | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| <i>Terschellingia</i> sp1 | 1A | 143 | 218 | 181 | 741 | 475 | 571 | 32 | 217 | 241 | 174 | 708 | 887 | 483 | 340 | 280 | 374 | 585 | 383 | 275 | 540 | 104 | 357 | 944 | 235 | 1060 | 215 | 67 | 119 | 154 | 301 |
| SE | | 69 | 32 | 110 | 369 | 205 | 203 | 12 | 105 | 178 | 38 | 210 | 398 | 325 | 116 | 68 | 49 | 419 | 23 | 65 | 219 | 29 | 116 | 413 | 91 | 457 | 52 | 24 | 56 | 44 | 19 |
| <i>Paracomesoma</i> | 1B | 363 | 218 | 64 | 407 | 662 | 478 | 20 | 66 | 29 | 116 | 336 | 773 | 135 | 316 | 453 | 530 | 753 | 230 | 520 | 646 | 42 | 644 | 350 | 254 | 352 | 322 | 81 | 621 | 103 | 619 |
| SE | | 131 | 53 | 27 | 187 | 54 | 233 | 7 | 39 | 11 | 9 | 186 | 127 | 18 | 30 | 26 | 188 | 244 | 91 | 169 | 64 | 16 | 274 | 173 | 111 | 51 | 110 | 16 | 144 | 17 | 58 |
| <i>Odontophora</i> | 1B | 187 | 200 | 98 | 432 | 274 | 278 | 70 | 209 | 239 | 55 | 210 | 341 | 138 | 212 | 220 | 235 | 217 | 192 | 113 | 88 | 69 | 93 | 243 | 72 | 165 | 67 | 92 | 171 | 18 | 166 |
| SE | | 53 | 22 | 23 | 280 | 54 | 33 | 40 | 72 | 196 | 8 | 82 | 126 | 75 | 60 | 35 | 66 | 77 | 15 | 47 | 14 | 31 | 20 | 141 | 26 | 41 | 23 | 14 | 69 | 4 | 26 |
| <i>Spirinia</i> | 2A | 133 | 134 | 269 | 31 | 195 | 179 | 149 | 419 | 50 | 5 | 82 | 120 | 201 | 65 | 316 | 26 | 13 | 352 | 116 | 108 | 122 | 20 | 35 | 44 | 57 | 221 | 64 | 21 | 31 | 0 |
| SE | | 74 | 20 | 88 | 16 | 79 | 55 | 23 | 110 | 1 | 3 | 82 | 82 | 50 | 21 | 109 | 26 | 13 | 148 | 47 | 56 | 23 | 11 | 17 | 23 | 20 | 174 | 13 | 12 | 28 | 0 |
| <i>Ptycholaimellus</i> | 2A | 116 | 66 | 22 | 146 | 194 | 76 | 68 | 52 | 57 | 221 | 362 | 438 | 14 | 25 | 14 | 84 | 190 | 93 | 7 | 0 | 65 | 98 | 208 | 131 | 32 | 21 | 73 | 24 | 18 | 123 |
| SE | | 7 | 25 | 9 | 82 | 65 | 52 | 21 | 9 | 11 | 114 | 235 | 147 | 8 | 22 | 9 | 49 | 88 | 35 | 7 | 0 | 25 | 15 | 101 | 43 | 26 | 1 | 16 | 12 | 7 | 23 |
| <i>Linhomoeus</i> | 2A | 4 | 4 | 15 | 33 | 65 | 1037 | 5 | 18 | 34 | 51 | 72 | 213 | 10 | 19 | 19 | 263 | 303 | 142 | 7 | 13 | 17 | 13 | 86 | 97 | 60 | 21 | 6 | 65 | 42 | 85 |
| SE | | 4 | 4 | 12 | 32 | 21 | 451 | 2 | 11 | 9 | 21 | 31 | 100 | 6 | 10 | 9 | 80 | 230 | 70 | 4 | 7 | 4 | 7 | 45 | 39 | 29 | 9 | 1 | 28 | 13 | 36 |
| <i>Sabatieria</i> | 1B | 6 | 9 | 7 | 30 | 56 | 284 | 12 | 22 | 26 | 57 | 168 | 107 | 58 | 82 | 11 | 226 | 151 | 63 | 23 | 40 | 17 | 84 | 479 | 89 | 34 | 11 | 9 | 38 | 112 | 212 |
| SE | | 6 | 9 | 6 | 28 | 30 | 73 | 2 | 14 | 13 | 13 | 79 | 50 | 17 | 42 | 11 | 93 | 45 | 17 | 19 | 20 | 9 | 17 | 246 | 15 | 17 | 4 | 6 | 13 | 20 | 61 |
| <i>Metachromadora</i> | 2B | 11 | 14 | 1 | 39 | 180 | 94 | 0 | 14 | 16 | 39 | 93 | 124 | 7 | 11 | 9 | 334 | 329 | 46 | 96 | 0 | 5 | 7 | 291 | 43 | 223 | 14 | 0 | 18 | 21 | 48 |
| SE | | 7 | 8 | 1 | 38 | 180 | 94 | 0 | 9 | 9 | 12 | 20 | 80 | 7 | 11 | 9 | 158 | 127 | 27 | 66 | 0 | 5 | 7 | 137 | 9 | 94 | 2 | 0 | 7 | 13 | 28 |
| <i>Sphaerolaimus</i> sp1 | 2B | 26 | 42 | 37 | 20 | 42 | 126 | 6 | 56 | 66 | 24 | 74 | 13 | 99 | 119 | 20 | 55 | 109 | 50 | 26 | 51 | 14 | 44 | 86 | 48 | 85 | 148 | 28 | 47 | 32 | 69 |
| SE | | 8 | 15 | 24 | 10 | 28 | 21 | 13 | 5 | 20 | 19 | 29 | 13 | 64 | 52 | 5 | 7 | 98 | 16 | 16 | 5 | 4 | 11 | 26 | 3 | 18 | 64 | 15 | 13 | 12 | 15 |
| <i>Axonolaimus</i> | 1B | 35 | 57 | 39 | 0 | 53 | 54 | 8 | 35 | 89 | 3 | 34 | 27 | 55 | 102 | 30 | 92 | 76 | 20 | 82 | 21 | 10 | 17 | 46 | 20 | 115 | 77 | 40 | 97 | 35 | 105 |
| SE | | 16 | 22 | 16 | 0 | 21 | 8 | 4 | 18 | 17 | 3 | 19 | 27 | 44 | 45 | 10 | 53 | 25 | 10 | 42 | 13 | 4 | 3 | 10 | 4 | 42 | 19 | 15 | 38 | 12 | 28 |
| <i>Daptonema</i> sp1 | 1B | 38 | 32 | 18 | 44 | 283 | 120 | 18 | 18 | 10 | 34 | 14 | 43 | 6 | 8 | 14 | 15 | 13 | 74 | 10 | 22 | 49 | 46 | 55 | 66 | 43 | 8 | 55 | 139 | 30 | 62 |
| SE | | 19 | 13 | 4 | 43 | 127 | 74 | 16 | 9 | 4 | 14 | 14 | 24 | 6 | 5 | 7 | 15 | 13 | 19 | 6 | 15 | 11 | 26 | 25 | 29 | 24 | 4 | 16 | 70 | 14 | 27 |
| <i>Paracatytholaimus</i> | 2A | 25 | 74 | 19 | 43 | 371 | 87 | 1 | 10 | 2 | 0 | 33 | 4 | 0 | 6 | 0 | 47 | 30 | 46 | 0 | 9 | 29 | 33 | 58 | 12 | 0 | 8 | 18 | 112 | 33 | 182 |
| SE | | 15 | 27 | 12 | 43 | 154 | 51 | 1 | 10 | 2 | 0 | 25 | 4 | 0 | 6 | 0 | 13 | 25 | 27 | 0 | 5 | 18 | 18 | 21 | 1 | 0 | 6 | 5 | 39 | 18 | 49 |
| <i>Metalinhomoeus</i> | 1B | 21 | 4 | 40 | 30 | 35 | 536 | 0 | 0 | 0 | 4 | 0 | 0 | 3 | 0 | 5 | 98 | 68 | 28 | 30 | 5 | 6 | 26 | 102 | 28 | 0 | 0 | 0 | 0 | 0 | 20 |
| SE | | 16 | 4 | 20 | 16 | 35 | 494 | 0 | 0 | 0 | 4 | 0 | 0 | 3 | 0 | 5 | 49 | 48 | 21 | 16 | 5 | 1 | 26 | 72 | 28 | 0 | 0 | 0 | 0 | 0 | 11 |
| <i>Eleutherolaimus</i> | 1B | 0 | 0 | 1 | 0 | 44 | 86 | 0 | 0 | 14 | 30 | 34 | 31 | 0 | 0 | 0 | 353 | 74 | 36 | 2 | 4 | 24 | 0 | 42 | 98 | 0 | 0 | 2 | 25 | 10 | 31 |
| SE | | 0 | 0 | 1 | 0 | 31 | 9 | 0 | 0 | 14 | 16 | 25 | 25 | 0 | 0 | 0 | 38 | 4 | 29 | 2 | 4 | 24 | 0 | 7 | 57 | 0 | 0 | 2 | 11 | 5 | 8 |
| <i>Atrochomadora</i> | 2A | 38 | 84 | 72 | 71 | 75 | 29 | 8 | 25 | 41 | 0 | 149 | 0 | 7 | 82 | 19 | 0 | 0 | 5 | 23 | 5 | 5 | 13 | 41 | 23 | 23 | 1 | 4 | 10 | 14 | 15 |
| SE | | 23 | 16 | 31 | 41 | 14 | 15 | 5 | 17 | 31 | 0 | 72 | 0 | 7 | 38 | 13 | 0 | 0 | 5 | 19 | 5 | 2 | 9 | 30 | 6 | 17 | 1 | 2 | 10 | 4 | 8 |
| <i>Daptonema</i> sp3 | 1B | 23 | 18 | 29 | 22 | 20 | 14 | 2 | 9 | 7 | 19 | 55 | 139 | 36 | 59 | 20 | 0 | 0 | 0 | 11 | 30 | 8 | 20 | 22 | 58 | 37 | 45 | 15 | 32 | 30 | 55 |
| SE | | 11 | 9 | 14 | 21 | 10 | 14 | 1 | 5 | 5 | 16 | 40 | 68 | 33 | 56 | 7 | 0 | 0 | 0 | 6 | 17 | 8 | 10 | 16 | 18 | 6 | 16 | 6 | 26 | 15 | 34 |
| <i>Linhomoeus</i> sp3 | 2A | 10 | 4 | 16 | 21 | 22 | 94 | 12 | 80 | 55 | 0 | 62 | 108 | 0 | 25 | 23 | 33 | 27 | 77 | 7 | 18 | 10 | 10 | 59 | 12 | 0 | 8 | 3 | 19 | 12 | 0 |
| SE | | 5 | 4 | 13 | 10 | 11 | 94 | 7 | 41 | 23 | 0 | 52 | 108 | 0 | 9 | 12 | 16 | 27 | 32 | 7 | 12 | 4 | 6 | 46 | 7 | 0 | 6 | 2 | 19 | 6 | 0 |
| <i>Metalinhomoeus</i> sp3 | 1B | 13 | 37 | 55 | 20 | 0 | 17 | 27 | 221 | 48 | 0 | 0 | 54 | 21 | 89 | 23 | 0 | 13 | 19 | 9 | 28 | 37 | 7 | 9 | 4 | 15 | 19 | 2 | 16 | 22 | 0 |
| SE | | 7 | 21 | 23 | 10 | 0 | 17 | 16 | 89 | 40 | 0 | 0 | 54 | 11 | 49 | 12 | 0 | 13 | 2 | 6 | 14 | 21 | 7 | 9 | 4 | 7 | 9 | 5 | 8 | 22 | 0 |
| <i>Oncholaimellus</i> | 2B | 8 | 19 | 34 | 21 | 21 | 60 | 10 | 34 | 33 | 19 | 62 | 68 | 0 | 25 | 10 | 60 | 4 | 34 | 19 | 56 | 15 | 10 | 21 | 8 | 17 | 8 | 33 | 11 | 2 | 54 |
| SE | | 8 | 5 | 16 | 21 | 21 | 21 | 3 | 9 | 4 | 8 | 25 | 36 | 0 | 5 | 5 | 40 | 4 | 14 | 1 | 28 | 5 | 6 | 15 | 4 | 9 | 4 | 7 | 6 | 2 | 44 |
| <i>Metalinhomoeus</i> sp4 | 1B | 23 | 84 | 87 | 20 | 12 | 48 | 23 | 179 | 11 | 0 | 0 | 0 | 14 | 36 | 34 | 0 | 0 | 25 | 2 | 64 | 15 | 7 | 0 | 4 | 17 | 18 | 5 | 0 | 0 | 7 |
| SE | | 11 | 39 | 40 | 19 | 12 | 29 | 15 | 71 | 11 | 0 | 0 | 0 | 10 | 8 | 13 | 0 | 0 | 6 | 2 | 35 | 3 | 7 | 0 | 4 | 9 | 18 | 5 | 0 | 0 | 7 |
| <i>Viscosia</i> | 2B | 9 | 9 | 51 | 0 | 21 | 136 | 32 | 19 | 13 | 0 | 0 | 18 | 12 | 0 | 0 | 69 | 59 | 34 | 10 | 29 | 11 | 0 | 34 | 39 | 10 | 10 | 23 | 24 | 7 | 20 |
| SE | | 5 | 9 | 41 | 0 | 11 | 59 | 9 | 19 | 13 | 0 | 0 | 12 | 6 | 0 | 0 | 34 | 19 | 14 | 5 | 15 | 3 | 0 | 8 | 25 | 7 | 7 | 13 | 12 | 7 | 14 |
| <i>Bathylaimus</i> | 1B | 13 | 0 | 7 | 0 | 0 | 228 | 1 | 0 | 14 | 3 | 14 | 35 | 7 | 0 | 0 | 107 | 0 | 14 | 0 | 7 | 6 | 10 | 4 | 0 | 0 | 9 | 2 | 2 | 12 | 16 |
| SE | | 7 | 0 | 7 | 0 | 0 | 26 | 1 | 0 | 2 | 3 | 14 | 6 | 7 | 0 | 0 | 45 | 0 | 7 | 0 | 7 | 1 | 6 | 4 | 0 | 0 | 5 | 2 | 2 | 4 | 8 |
| <i>Microlaimus</i> | 1B | 4 | 14 | 0 | 10 | 46 | 12 | 3 | 12 | 11 | 0 | 39 | 68 | 0 | 0 | 14 | 54 | 27 | 78 | 3 | 0 | 6 | 13 | 38 | 0 | 0 | 5 | 5 | 0 | 0 | 0 |
| SE | | 4 | 14 | 0 | 9 | 46 | 12 | 3 | 6 | 5 | 0 | 22 | 68 | 0 | 0 | 7 | 19 | 27 | 42 | 3 | 0 | 1 | 9 | 7 | 0 | 0 | 3 | 5 | 0 | 0 | 0 |
| <i>Promonhystera</i> | 1B | 35 | 23 | 15 | 21 | 31 | 12 | 0 | 0 | 12 | 5 | 20 | 13 | 3 | 0 | 0 | 49 | 0 | 0 | 18 | 21 | 6 | 10 | 17 | 15 | 0 | 15 | 6 | 9 | 21 | 40 |
| SE | | 9 | 5 | 6 | 10 | 18 | 12 | 0 | 0 | 10 | 3 | 12 | 13 | 3 | 0 | 0 | 16 | 0 | 0 | 11 | 13 | 3 | 6 | 17 | 9 | 0 | 2 | 3 | 6 | 13 | 15 |
| <i>Halalaimus</i> | 1A | 26 | 0 | 4 | 0 | 21 | 74 | 1 | 16 | 26 | 0 | 12 | 14 | 2 | | | | | | | | | | | | | | | | | |

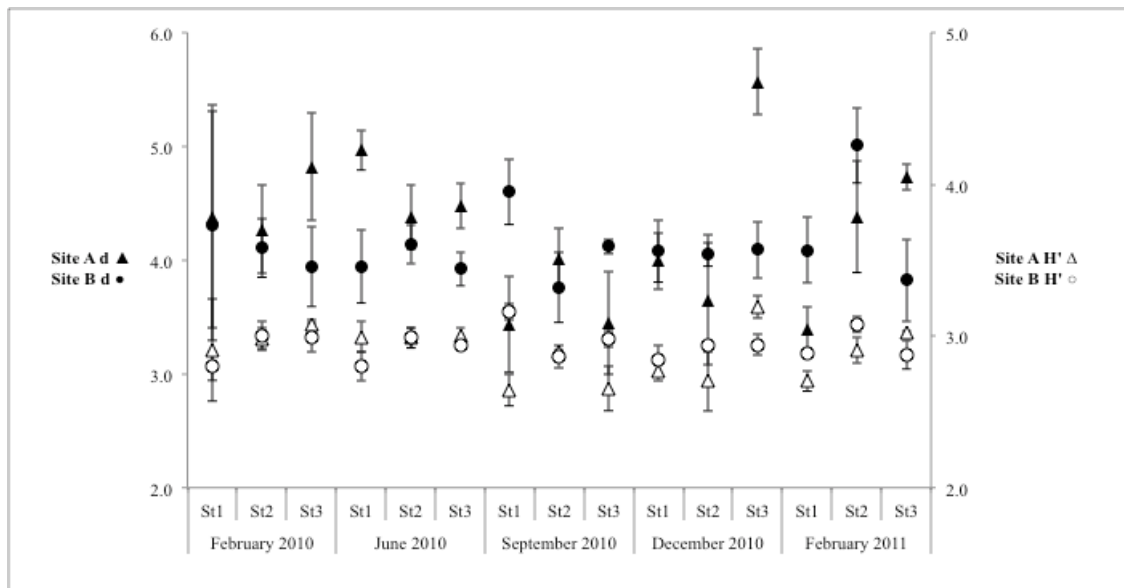


Figure 6. Margalef Index ($d \pm$ standard error) and Shannon–Wiener values ($H' \pm$ standard error), average values in Stations (St1, St2, St3) at Sites (A and B) and Sampling occasions (February 2010, June 2010, September 2010, December 2010 and February 2011).

September 2010 ($35.8 \pm 5.9\%$), December 2010 ($43.0 \pm 8.9\%$) and February 2011 (43.5 ± 7.8). Epigrowth feeders were the most abundant feeding group in February ($42.5 \pm 8.8\%$) and in June 2010 ($38.9 \pm 6.8\%$). The highest contribution of selective deposit feeders (1A) was in December 2010 at site B ($25.5 \pm 7.8\%$) and in February 2011 at site A ($31.6 \pm 13.8\%$). The highest contribution of omnivores/predators (2B) was in February 2011 ($14.2 \pm 3.4\%$) (Fig. 5). PERMANOVA analysis of the trophic structure data only showed significant differences between sites (factor “Site”, $p < 0.05$) and significant interactions between factor “Site”, “Station” and “Sampling occasion” ($p < 0.05$) (Table 2). Individual pairwise comparisons on the interaction factor revealed a low variability among sampling occasions and between stations, although some significant differences were detected. At site A, there were significant differences in

Table 2. Results of the three-factor PERMANOVA test “Site” A and B (2 levels, random), “Sampling occasion” February 2010, June 2010, September 2010, December 2010 and February 2011 (5 levels, fixed) and “Station” St1, St2 , St3 (3 levels, nested in Site), for all univariate descriptors of nematode density, assemblage composition and diversity. Values in bold indicate significant effects ($p < 0.05$).

| Source of variation | | Degrees of freedom | Sum of squares | Mean squares | Pseudo-F | P(perm) | perms | P(Monte Carlo) |
|----------------------------|------------------------------------|--------------------|----------------|--------------|----------|---------------|-------|----------------|
| Nematodes total densiy | | | | | | | | |
| | Site | 1 | 14170 | 14170 | 47 068 | 0.0001 | 9916 | 0.0004 |
| | Sampling occasion | 4 | 12328 | 3082 | 1.81 | 0.0172 | 7262 | 0.0157 |
| | Station (Site) | 4 | 6527.7 | 1631.9 | 10 698 | 0.3564 | 9874 | 0.3469 |
| | Site x Sampling Occasion | 4 | 6811.2 | 1702.8 | 11 163 | 0.2962 | 9866 | 0.2834 |
| | Station (Site) x Sampling occasion | 16 | 24407 | 1525.4 | 19 526 | 0.0001 | 9722 | 0.0001 |
| | Residual | 60 | 46875 | 781.26 | | | | |
| | Total | 89 | 1111200000.0 | | | | | |
| Trophic composition | | | | | | | | |
| | Site | 1 | 3523.3 | 3523.3 | 4.0855 | 0.0206 | 9955 | 0.037 |
| | Sampling occasion | 4 | 2452.3 | 613.06 | 1.7299 | 0.1566 | 7252 | 0.2011 |
| | Station (Site) | 4 | 2441.6 | 610.41 | 1.4589 | 0.2329 | 9957 | 0.2093 |
| | Site x Sampling Occasion | 4 | 1417.6 | 354.39 | 0.84702 | 0.5471 | 9954 | 0.5577 |
| | Station (Site) x Sampling occasion | 16 | 6694.3 | 418.39 | 1.9104 | 0.0069 | 9904 | 0.0093 |
| | Residual | 60 | 13141 | 219.01 | | | | |
| | Total | 89 | 29670 | | | | | |
| Margalef index | | | | | | | | |
| | Site | 1 | 6.3181 | 6.3181 | 0.48724 | 0.7933 | 9952 | 0.8823 |
| | Sampling occasion | 4 | 77.564 | 19.391 | 0.56086 | 0.7699 | 7262 | 0.7145 |
| | Station (Site) | 4 | 115.23 | 28.807 | 1.1728 | 0.3589 | 9954 | 0.3725 |
| | Site x Sampling Occasion | 4 | 138.29 | 34.574 | 1.4075 | 0.2794 | 9956 | 0.2826 |
| | Station (Site) x Sampling occasion | 16 | 393.01 | 24.563 | 1.3626 | 0.1907 | 9937 | 0.1817 |
| | Residual | 60 | 1081.6 | 18.027 | | | | |
| | Total | 89 | 1812 | | | | | |
| Shannon-Wiener index | | | | | | | | |
| | Site | 1 | 4.3271 | 4.3271 | 0.64295 | 0.6674 | 9953 | 0.6217 |
| | Sampling occasion | 4 | 8.4632 | 2.1158 | 0.31996 | 0.8301 | 7304 | 0.8501 |
| | Station (Site) | 4 | 24.057 | 6.0142 | 1.5863 | 0.2239 | 9957 | 0.236 |
| | Site x Sampling Occasion | 4 | 26.451 | 6.6127 | 1.7441 | 0.1891 | 9942 | 0.1914 |
| | Station (Site) x Sampling occasion | 16 | 60.662 | 3.7913 | 1.5093 | 0.1228 | 9937 | 0.1279 |
| | Residual | 60 | 150.72 | 2.512 | | | | |
| | Total | 89 | 274.68 | | | | | |
| Index of trophic diversity | | | | | | | | |
| | Site | 1 | 0.41303 | 0.41303 | 0.15573 | 0.9946 | 9951 | 0.9987 |
| | Sampling occasion | 4 | 26.946 | 6.7364 | 0.12248 | 0.9909 | 7279 | 0.9707 |
| | Station (Site) | 4 | 52.386 | 13.096 | 1.285 | 0.3194 | 9953 | 0.3232 |
| | Site x Sampling Occasion | 4 | 219.99 | 54.998 | 5.3965 | 0.0063 | 9948 | 0.0057 |
| | Station (Site) x Sampling occasion | 16 | 163.06 | 10.192 | 0.90603 | 0.5694 | 9905 | 0.5679 |
| | Residual | 60 | 674.92 | 11.249 | | | | |
| | Total | 89 | 1137.7 | | | | | |
| Maturity Index | | | | | | | | |
| | Site | 1 | 57 844 | 57 844 | 25 756 | 0.001 | 997 | 0.001 |
| | Sampling occasion | 4 | 66 576 | 16 644 | 0.55479 | 0.692 | 999 | 0.722 |
| | Station (Site) | 6 | 18 771 | 31 285 | 2 816 | 0.014 | 999 | 0.029 |
| | Site x Sampling Occasion | 4 | 12 | 3 | 14 718 | 0.238 | 999 | 0.26 |
| | Station (Site) x Sampling occasion | 14 | 28 536 | 20 383 | 18 347 | 0.053 | 999 | 0.052 |
| | Residual | 60 | 66 659 | 1 111 | | | | |
| | Total | 89 | 185.09 | | | | | |

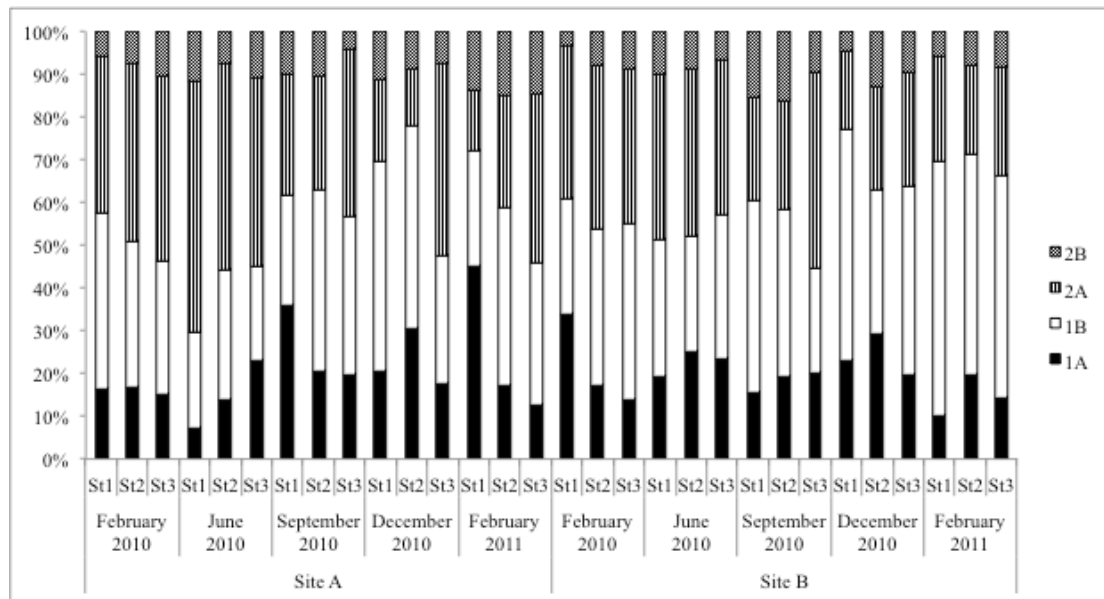


Figure 5. Percentage of contribution of four different trophic groups (1A – selective deposit feeders; 1B – non-selective deposit feeders; 2A – epistrate feeders; 2B – predators), average values in Stations (St1, St2, St3) at Sites (A and B) and Sampling occasions (February 2010, June 2010, September 2010, December 2010 and February 2011).

June 2010 between station 1 and 2 ($p < 0.05$), in December 2010 between station 2 and 3 ($p < 0.05$) and in February 2011 between station 1 and 3 ($p < 0.05$). At site B, there were significant differences in June 2010 between station 1 and 2 and between station 1 and 3 ($p < 0.05$), and in February 2011 between station 2 and 3 ($p < 0.05$).

The average of trophic diversity (ITD) values were high (Site A, mean = 2.98 ± 0.05 ; Site B, mean = 3.00 ± 0.07) indicating a trophic diversity (Fig. 6). PERMANOVA analysis of the ITD did not detect any significant differences between sites, stations or sampling occasions.

The Maturity Index (MI) at site A ranged from 2.32 ± 0.12 (Station 1, February 2010) to 2.61 ± 0.02 (Station 1, September 2010). At site B, ranged from 2.16 ± 0.01 (Station 1, February 2011) to 2.51 ± 0.07 (Station 1, February 2010) and most nematode species showed *c-p* score of 2 (58%) described by Bongers & Bongers (1998) as ‘general

opportunists' followed by *c-p* score of 3 (29%) and 4 (13%) (Fig. 6). PERMANOVA analysis of MI index revealed significant differences between sites (factor "Site", $p < 0.05$) and between stations (factor "Stations", $p < 0.05$), but did not show any significant interaction effect (Table 2).

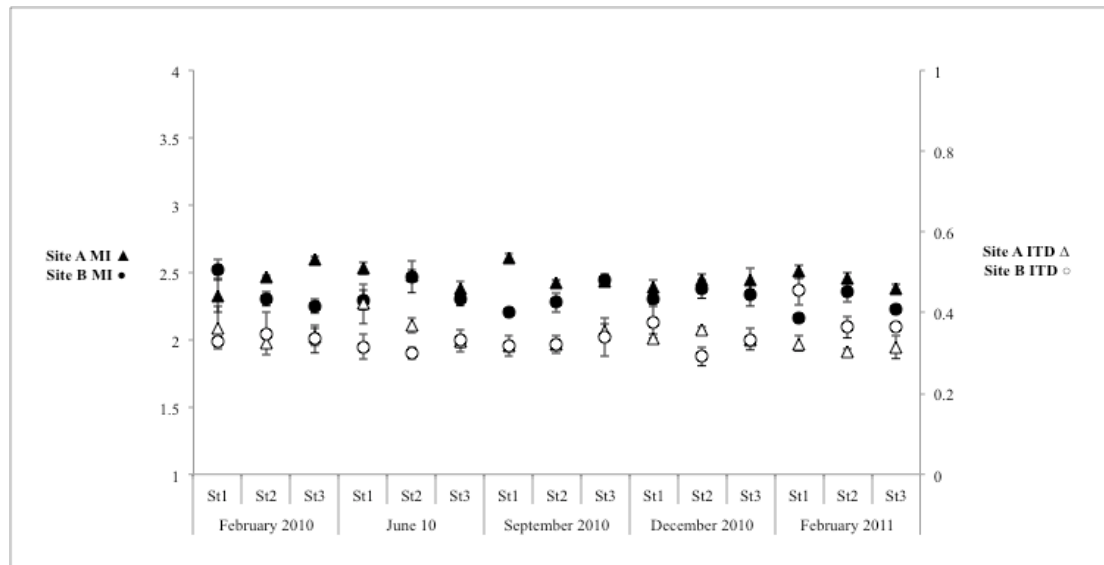


Figure 6. Index of Trophic Diversity (ITD \pm standard error) and Maturity Index (MI \pm standard error), average values in Stations (St1, St2, St3) at Sites (A and B) and Sampling occasions (February 2010, June 2010, September 2010, December 2010 and February 2011).

Nematode assemblage composition

PERMANOVA analysis of the density data (individuals 10 cm^{-2}) of nematode assemblages showed significant differences between sampling occasions (factor "Sampling occasions", $p < 0.05$), revealing consistently lower densities at site A during the sampling occasions, although the variability among sampling occasions within each site was low. Additionally, PERMANOVA analysis showed significant differences between sites (factor "Site", $p < 0.05$). Interactions between factors "Site", "Station" and "Sampling occasion" were revealed, although no interactions were obtained

between factor “Site” and “Sampling occasion” (Table 2). Individual pairwise comparisons on interaction factor (“Site”, “Station” and “Sampling occasion”), showed at site A, significant differences in December 2010 between station 2 and 3 ($p < 0.05$) and in February 2011 between station 1 and 2 ($p < 0.05$). At site B, individual pairwise comparisons showed significant differences in September 2010 between station 1 and 3 ($p < 0.05$) and in February 2011 between station 2 and 3 ($p < 0.05$). These results, supported by the PCO ordination plot, clearly reflect a distinct pattern between sites A and B and temporal differences between sites. Further, it is visible the low variability of the nematode communities within stations on each site and sampling occasions (Fig. 7).

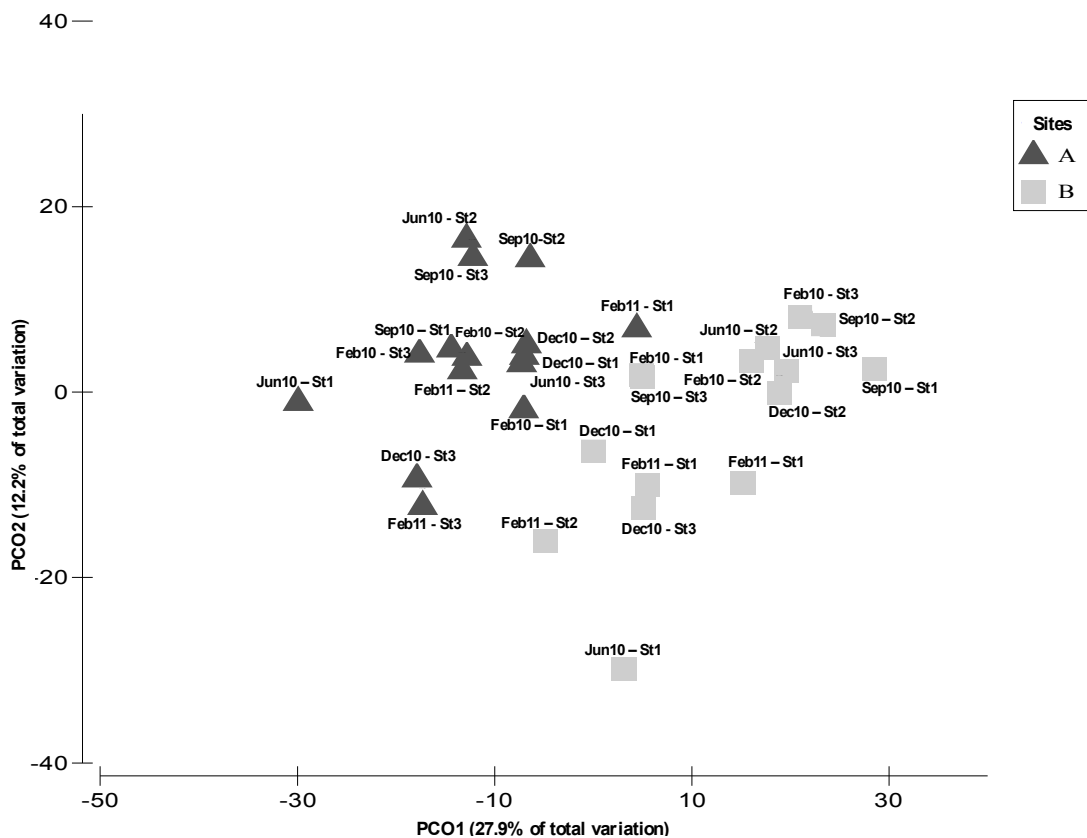


Figure 7. Principal coordinates analysis (PCO) based on nematode density dataset in each “Site” A and B (2 levels, random), “Sampling occasion” February 2010, June 2010, September

2010, December 2010 and February 2011 (5 levels, fixed) and “Station” St1, St2 , St3 (3 levels, nested in Site). PCO1 = 27.9%, PCO2 = 12.2%.

The SIMPER analysis showed how nematode genera contributed to similarity values of the *a priori* defined groups. The genera that most contributed to the similarity within site A were *Terschellingia* sp.1, *Paracomesoma*, *Spirinia* and *Odontophora*, while at site B they were *Paracomesoma*, *Terschellingia* sp.1, *Odontophora* and *Ptycholaimellus*. The genera that contributed most to the dissimilarities between sites A and B were *Terschellingia* sp.1, *Paracomesoma*, *Spirinia*, *Metachromadora* and *Linhomoeus*.

Separate BIOENV analyses were performed for each sampling occasion and sites, in order to analyse the main factors responsible for the distribution patterns of nematode communities throughout the period of study. The combination of four variables: nitrite (NO_2^-), nitrate (NO_3^-), phosphate (PO_4^{3-}) of the water above sediment (small pool) and biomass (AFDW) of *Z. noltii*, accounted for around 90% of the variability within nematode assemblages. However, only very low Spearman’s rank correlations were obtained ($\rho=0.268$).

Table 3. Results of SIMPER analysis indicating percentage (bold) similarity (Shaded boxes) and dissimilarity (Non-shaded boxes) between sites (A and B) and sampling occasions (February 2010, June 2010, September 2010, December 2010 and February 2011). The table also lists all nematode genera which contribute with at least 3.5%.

| | Site A | Site B | February 2010 | June 2010 | September 2010 | December 2010 | February 2011 |
|----------------|--|---|--|--|--|---|---|
| Site A | 58.2% <i>Terschellingia</i> sp1 <i>Paracomesoma</i> <i>Spirinia</i> <i>Odontophora</i> <i>Sphaerolaimus</i> sp1 <i>Axonolaimus</i> <i>Ptycholaimellus</i> | | | | | | |
| Site B | 50.6% <i>Terschellingia</i> sp1 <i>Paracomesoma</i> <i>Spirinia</i> <i>Metachromadora</i> <i>Linhomoeus</i> <i>Sabatieria</i> <i>Ptycholaimellus</i> | 55.8% <i>Paracomesoma</i> <i>Terschellingia</i> sp1 <i>Odontophora</i> <i>Ptycholaimellus</i> <i>Sabatieria</i> <i>Linhomoeus</i> <i>Sphaerolaimus</i> sp1 <i>Metachromadora</i> <i>Paracyatholaimus</i> <i>Daptonema</i> sp1 | | | | | |
| February 2010 | | | 53.5% <i>Paracomesoma</i> <i>Odontophora</i> <i>Terschellingia</i> sp1 <i>Spirinia</i> <i>Ptycholaimellus</i> <i>Atrochromadora</i> <i>Sphaerolaimus</i> sp1 <i>Daptonema</i> sp1 <i>Axonolaimus</i> <i>Paracyatholaimus</i> | | | | |
| June 2010 | | | 51.0% <i>Terschellingia</i> sp1 <i>Paracomesoma</i> <i>Odontophora</i> <i>Spirinia</i> <i>Paracyatholaimus</i> | 53.9% <i>Terschellingia</i> sp1 <i>Ptycholaimellus</i> <i>Odontophora</i> <i>Paracomesoma</i> <i>Spirinia</i> <i>Sabatieria</i> <i>Linhomoeus</i> <i>Oncholaimellus</i> <i>Sphaerolaimus</i> sp1 <i>Metachromadora</i> | 48.3% <i>Paracomesoma</i> <i>Terschellingia</i> sp1 <i>Spirinia</i> <i>Odontophora</i> <i>Ptycholaimellus</i> | 47.4% <i>Paracomesoma</i> <i>Terschellingia</i> sp1 <i>Odontophora</i> <i>Linhomoeus</i> <i>Ptycholaimellus</i> <i>Metachromadora</i> <i>Paracyatholaimus</i> <i>Spirinia</i> <i>Sabatieria</i> | 47.7% <i>Terschellingia</i> sp1 <i>Paracomesoma</i> <i>Ptycholaimellus</i> <i>Odontophora</i> <i>Spirinia</i> <i>Axonolaimus</i> |
| September 2010 | | | 47.4% <i>Terschellingia</i> sp1 <i>Paracomesoma</i> <i>Spirinia</i> <i>Linhomoeus</i> <i>Paracyatholaimus</i> <i>Metachromadora</i> | | 58.9% <i>Paracomesoma</i> <i>Terschellingia</i> sp1 <i>Odontophora</i> <i>Spirinia</i> <i>Sabatieria</i> <i>Sphaerolaimus</i> sp1 <i>Linhomoeus</i> <i>Axonolaimus</i> <i>Ptycholaimellus</i> | 43.6% <i>Paracomesoma</i> <i>Terschellingia</i> sp1 <i>Spirinia</i> <i>Metachromadora</i> <i>Odontophora</i> <i>Linhomoeus</i> | 44.9% <i>Terschellingia</i> sp1 <i>Paracomesoma</i> <i>Spirinia</i> <i>Metachromadora</i> <i>Odontophora</i> |
| December 2010 | | | 46.6% <i>Paracomesoma</i> <i>Terschellingia</i> sp1 <i>Odontophora</i> <i>Linhomoeus</i> <i>Ptycholaimellus</i> <i>Metachromadora</i> <i>Paracyatholaimus</i> <i>Spirinia</i> <i>Sabatieria</i> | | | 58.5% <i>Terschellingia</i> sp1 <i>Paracomesoma</i> <i>Odontophora</i> <i>Spirinia</i> <i>Sabatieria</i> <i>Sphaerolaimus</i> sp1 <i>Ptycholaimellus</i> <i>Daptonema</i> sp1 <i>Axonolaimus</i> | 41.8% <i>Paracomesoma</i> <i>Terschellingia</i> sp1 <i>Metachromadora</i> <i>Ptycholaimellus</i> <i>Spirinia</i> |
| February 2011 | | | 48% <i>Terschellingia</i> sp1 <i>Paracomesoma</i> <i>Spirinia</i> <i>Odontophora</i> <i>Linhomoeus</i> <i>Paracyatholaimus</i> <i>Metachromadora</i> | | | | 60.2% <i>Paracomesoma</i> <i>Terschellingia</i> sp1 <i>Odontophora</i> <i>Axonolaimus</i> <i>Sphaerolaimus</i> sp1 <i>Sabatieria</i> <i>Linhomoeus</i> <i>Spirinia</i> <i>Ptycholaimellus</i> <i>Daptonema</i> sp1 <i>Paracyatholaimus</i> <i>Daptonema</i> sp3 |

Discussion

Most restoration and recovery studies in estuarine and coastal waters have focused on benthic invertebrates (Borja et al. 2010), due to the value of using a sedentary component, that has the ability to reflect the quality of the environment conditions, and also because of the basic understanding of structure and dynamics of these taxonomic assemblages (Verdonschot et al. 2012). The changes of the benthic communities do not only affect the abundance of organisms and the dominance structure but also their temporal and spatial distribution patterns. Macrobenthic invertebrates have been traditionally used to assess the biological responses (Pinto et al. 2009), however meiofauna has several potential assessment advantages (Patrício et al. 2012). The rate and speed of the habitat recovery process is highly linked to turnover and the life span of the organisms and the meiofaunal recovery is faster than macrofauna (Borja et al. 2010).

The total disappearing of *Z. noltii* seagrass beds of Mira estuary was followed by a passive and natural recovery process as described by Elliot et al. (2007). This recovery is characterized by dense patches of vegetation alternating with very sparse or even non vegetation (bare muddy sediments), giving a strongly heterogeneous temporal and spatial distribution of seagrass beds. It is known that nematode assemblages respond to habitat variability, they have strongly heterogeneous distribution and horizontal patchiness is particularly pronounced. The physical factors are determinants generating macro-scale (e.g. km-scale) patchiness of the nematode assemblages, while food distribution and social or reproductive behaviour cause micro-scale (e.g. m-scale) heterogeneity (Li et al. 1997; Moens et al. 1999). Therefore, it was expected a high

temporal and spatial variability of the nematode abundance, diversity and trophic composition between sites, stations and sampling occasions.

The environmental variables measured give an indication of the similar environmental conditions and patterns for both sites and sampling occasions, although there is a lack data at station levels in each site. At site A, the environmental conditions in February 2011 were clearly different which could be explained by the increase of grain size of the sediments and high values of chlorophyll *a*. Nutrients concentration remained constant at both sites along the study period, which is likely explained by the absence of significant anthropogenic impacts due to the location of this estuary in a protected area. The causes of *Z. noltii* collapse are still not determined, the absence of visible anthropogenic pressures allowed to relate spatial and temporal patterns of the environmental variables measured mainly with the natural stressors' characteristics of this estuarine system, such as: *i*) its morphology, since the terminal section of the river is rather regular and facilitates the upstream tidal penetration, and *ii*) a normally reduced outflow determined by the region's annual rainfall distribution (concentrated between January and March) with the rest of the year being usually dry (Paula et al. 2006). Added to natural structural features of this estuarine system, in the last decade important changes of the sedimentary dynamics, which may lead to a seriously drastic impact on seagrass meadows (Cabaço et al. 2008), have been observed (Adão personal communication). In fact, at both sampling sites, there were obtained higher proportions of silt and clay prior to the total loss of *Z. noltii* (Adão 2004), although during this early recovery process an increase of the proportions of the mean sand and coarse sediments was registered.

The biomass of *Z. noltii* throughout the study was very low, which is clearly explained by this intermittent recovery of the seagrass beds, characterized by the presence of the

highest values in June 2010 (site A) and February 2010 (site B), alternating with the lowest values in February 2010 (site A) and September 2010 (site B) or even no vegetation in December 2010 (site A) and June 2010 (site B). The temporal and horizontal spatial distribution of the seagrass beds became strongly heterogeneous and the area covered by the vegetation after the collapse was clearly smaller than before (Cunha et al. 2013).

The environmental conditions observed at both sites could be described as typical of the intertidal muddy sediments of the estuarine euhaline section, reflecting a strong dependence on the marine environment with high salinity and high fractions of silt and clay (Teixeira et al. 2008). Although the results obtained are at sampling site level, they provide important information about the ecological conditions based on main factors driving temporal and spatial distribution, density, and species composition of free-living nematodes, namely salinity, sediment grain size composition (Austen & Warwick 1989; Soetaert et al. 1995; Steyaert et al. 2003; Adao et al. 2009; Alves et al. 2013) and the vegetation presence (Bell et al. 2001).

The nematode densities and the number of species obtained were generally high and comparable to several estuarine intertidal muddy sediments located in the euhaline areas of the estuaries (Austen et al. 1989; Soetaert et al. 1994; Soetaert et al. 1995; Schizas & Shirley 1996; Coull 1999; Steyaert et al. 2003; Rzeznik-Orignac et al. 2003; Fonseca et al. 2011). The euhaline area of the Mira estuary is usually characterized by the highest values of salinity, proportions of silt and clay, organic matter content and food availability, which are recognized as the main factors that influence nematode species density and diversity distribution (Li et al. 1997; Coull 1999; Adao et al. 2009; Alves et al. 2013).

The genera composition and the dominance of species obtained were also typical of the nematode assemblages from estuarine intertidal sediments, the species identified are commonly cited in the literature as mud-adapted, characterized by higher densities of the genera belonging to the families Linhomoeidae (*Terschellingia*, *Linhomoeus*), Comesomatidae (*Paracomesoma*), Desmodoridae (*Spirinia*) and Axonolaimidae (*Odontophora*) (Wieser 1960; Tietjen 1969; Soetaert et al. 1995; Austen et al. 1989; Smol et al. 1994; Ólafsson et al. 2000; Fisher & Sheaves 2003; Rzeznik-Orignac et al. 2003; Steyaert et al. 2003; Johnson et al. 2007; Fonseca et al. 2011; Moens et al. 2013). These genera share common characteristics such as, tolerance to hypoxic conditions (Jensen 1984; Steyaert et al. 2007) and body morphology that may be advantageous to glide through and over the fine sediment (Warwick 1971), becoming typical in estuarine muddy sediments (Heip et al. 1985). The two most abundant genera registered in this study, *Terschellingia* and *Paracomesoma*, are able to thrive in natural and anthropogenic disturbed habitats (Steyaert et al. 2007; Gambi et al. 2009; Armenteros et al. 2009; Alves et al. 2013), including extreme conditions (Moreno et al. 2008; Fonseca et al. 2011).

No significant differences were found for species richness and structural diversity based on Margalef Index (d) and Shannon–Wiener Index (H') between sites, stations or sampling occasions. This result was not anticipated, we would expect high variability in the number of species due to the horizontal heterogeneity as a result of the sparse nature of the seagrass beds recovery, small patches alternating with bare sediment spatially and temporally. It is important to note that despite several studies that support higher level of biodiversity and abundance of organisms in sediments of the seagrass beds than in the surrounding bare sediment (Heck et al. 1995; Connolly 1997; Hirst & Attrill 2008), some studies that focused on the effect of habitat modification caused by the presence of

Z. noltii reported no differences on the diversity and taxa richness between vegetated and unvegetated sediments (Aryuthaka & Kikuchi 1996; Ndaró & Olafsson 1999; Fonseca et al. 2011).

The trophic composition of nematode communities showed significant differences between sampling sites, clearly explained by the highest densities at site B of the trophic groups. The nematode assemblages were dominated by non-selective deposit feeders (1B) and epigrowth feeders (2A) at both sites. This was expected in intertidal muddy sediments and also in seagrass sediments (Bouwman et al. 1984; Escaravage et al. 1989; Aryuthaka & Kikuchi 1996; Danovaro et al. 2002; Rzeznik-Orignac et al. 2003). Epigrowth feeders (2A) are frequently the dominant trophic group in seagrass beds. These plants tend to enter in the food web mainly as detritus and support a diverse epiphyte community, that is heavily grazed by small invertebrates, such as benthic nematodes. Microphytobenthos (MPB) are another important food source, which often exhibit high production rates in seagrass beds, being available for consumption and easy digestible (Fisher & Sheaves 2003; Danovaro et al. 2002; Fonseca et al. 2011). Using dual stable isotope signatures, the food web structure was examined at both sites (A and B) by comparing the food sources of macrobenthos and meiobenthos (nematodes and harpacticoid copepods at genus/species level) in the seagrass patches *versus* adjacent unvegetated sediments. The organic carbon input for the diet of estuarine macrobenthos and meiobenthos during the early recovery derives from various sources: seagrass detritus, microphytobenthos, epiphytic microalgae and suspended particulate organic matter (Vafeiadou et al. 2013). The MPB revealed to be among the main resources of most nematode taxa in *Z. noltii* patches and in surrounding bare mud demonstrating that seagrass-associated inputs extend beyond the borders of the vegetation patches (Vafeiadou et al. 2014). The absence of temporal patterns of the assemblage's trophic

composition could be caused by the permanent availability of the resources. The examination of the resource use and trophic position of nematodes and harpacticoid copepods at both sites also revealed no significant differences between June 2010 and February 2011 (Vafeiadou et al. 2013; Vafeiadou et al. 2014).

Coupled with taxonomic diversity, functional diversity is important for interpreting distribution patterns of communities (Schratzberger et al. 2008). Specific indicators relying on nematodes information, such as the Trophic Diversity Index (ITD⁻¹) and the Maturity Index (MI) behaved differently: the high values of the ITD represented high trophic diversity (Moreno et al. 2011), though the low values of MI suggest disturbed habitats, since the opportunistic genera are dominant in adverse conditions (Bongers & Bongers 1998). In our study a high trophic diversity was obtained, indicating the nematode community responses to good ecological condition of the sediments. On the contrary, the MI results suggest disturbed habitat conditions, a clear dominance of “genera opportunists” (Bonger & Bongers 1998) with *c-p* 2, able to take advantage of disturbed and polluted environments (Gyedu-Ababio & Baird 2006). The sampling sites were located in the euhaline section of the estuary, which are highly naturally stressed because of the high degree of variability in their abiotic characteristics; therefore the structural features of the estuarine communities under this natural stress resemble those of the anthropogenic stressed areas as defined within the context of the “Estuarine Quality Paradox” (Dauvin & Ruellet 2009). The temporal and spatial patterns based on ITD values did not emerge, the MI results presented a pattern based on sampling sites, as a result of the density of the assemblages, although both sites presented the dominance of “genera opportunists”.

The most important spatial pattern emerged from the nematode density distribution between both sampling sites, most likely due to the influence of the highest density

registered at site B, located further from the estuary mouth than site A. The seagrass beds of both sampling sites were ecologically similar, the main environmental factors structuring nematode assemblages such as salinity, sediment composition (Ferrero et al. 2008; Adao et al. 2009; Alves et al. 2013) and the biomass of the seagrass beds (Fisher & Sheaves 2003) were also strongly similar. Nevertheless, site B is located further from the estuary mouth than site A, in a more protected area with low hydrodynamics and anthropogenic activity encompassing high nematode densities.

The low horizontal variability obtained among sampling stations was clearly revealed by no significant differences in nematode abundance, diversity and trophic composition. This result was not anticipated due to the increase of habitat heterogeneity imposed by the spatial and temporal irregular distribution of the small-sized seagrass patches recovery of *Z. noltii* seagrass beds, where the spatial variability of the nematodes assemblages would be expected to increase. Several studies have demonstrated that habitat heterogeneity has a determining role in the high variability of the nematode assemblages, which has a heterogeneous spatial distribution (Coull 1988; Soetaert et al. 1995; Li et al. 1997). The low spatial heterogeneity between stations could be understood by the results obtained in the study of food web structure, which demonstrated that seagrass associated inputs extend beyond the borders of the vegetation patches (Vafeiadou et al. 2014), supporting the low heterogeneity of nematode assemblages at stations levels.

No clear temporal patterns of the nematode density, trophic composition and diversity were observed, despite the small density differences detected within sampling occasions at each site. The temporal fluctuations of nematodes are regulated mainly by the seasonality of the temperature, salinity, sediment particle size, oxygen, availability of food resources, trophic interactions, predation, competition and the reproductive burst

of several species, which have been considered the major factors regulating the temporal patterns of nematodes inhabiting intertidal systems (Alongi 1987; Eskin & Coull 1987; Bouvy & Soyer 1989; Vincx 1989; Ansari & Parulekar 1993; Schizas & Shirley 1996; Ólafsson & Elmegren 1997; Steyaert et al. 1999; Adão 2004). The low temporal variability of the environmental factors and the absence of differences detected in isotope signatures of consumers among sampling occasions (Vafeiadou et al. 2013; Vafeiadou et al. 2014) are in agreement with the absence of a clear temporal pattern during this early recovery process.

Conclusion

In conclusion, the null hypothesis of this study was confirmed, the spatial and temporal distribution patterns of the nematode assemblages were not sensitive to the increase of the horizontal heterogeneity of the sediments provided by the passive natural recovery of *Z. noltii* in Mira estuary. The structuring environmental conditions driving the spatial and temporal variability of the nematode assemblage did not change significantly during the early recovery process. The nematode assemblages revealed ability to withstand the natural variability, providing distinctive assemblages typical of the intertidal sediments from euhaline section, adapting naturally to high stress conditions and presenting high density and diversity. The obtained baseline data allow us to understand the essence of the functional responses of nematode assemblages to a passive natural recovery process and that a good ecological status of the ecosystem can be achieved given the steadily recovery of seagrass beds in the Mira estuary.

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Chapter II

A comparative analysis of benthic nematodes assemblages before habitat loss and during the early recovery of *Zostera noltii* seagrass beds in Mira estuary (Southwest Coast of Portugal)

Abstract

Benthic nematodes are widely regarded as very suitable organisms to monitor potential ecological effects of natural and anthropogenic disturbances in aquatic ecosystems. During 2008, the stable seagrass beds of *Zostera noltii* located in Mira estuary (SW Portugal) disappeared completely. However, during 2009, slight symptoms of natural recovery were observed, a process which has since evolved intermittently. These seagrass beds have a rare database available in Portugal sampled before the disturbance based on temporal and spatial biodiversity patterns nematodes assemblages. The main goal was to investigate the responses of nematode assemblages to *Z. noltii* collapse, based on both communities before and during the early natural habitat recovery. We hypothesized that collapse would induce a decrease in abundance and both structural and functional diversity of the nematode assemblages. The comparison of these descriptors before and after the collapse, showed that nematode communities densities were significantly higher before the collapse, while after the collapse they demonstrated a natural adjustment to the new conditions with a higher diversity. Despite the significant differences found between sampling occasions, a temporal pattern was not evident. The nematodes community response following this extreme event exhibited considerable resistance and resilience to the new environmental conditions.

Keywords: Nematodes, *Zostera noltii*, natural recovery, stable condition, spatial and temporal distribution.

Introduction

Meiobenthic communities, especially nematodes, due to their ubiquitous distribution, varying from pristine to extremely polluted habitats, provide valuable information regarding ecosystems health (Sheppard 2006). Benthic nematodes are the most diverse and numerically dominant metazoans in aquatic habitats, which is clearly explained by their ecological characteristics such as small size, body morphology, fast reproduction, rapid life histories, presence across the food web and intimate association with sediments (Kennedy & Jacoby 1999; Schratzberger et al. 2000; Austen & Widdicombe 2006; Alves et al. 2013). These attributes give nematodes strong advantages over other potential indicators, as they can reflect changes in environmental conditions over small spatial scales through changes in density, diversity, structure and functioning, being informative in the assessment of estuarine and marine biological integrity (Norling et al. 2007; Danovaro et al. 2008; Patrício et al. 2012). The small changes in sediment structure, chemistry, disturbance and potential food, such as bacteria and microphytobenthos, are closely linked to nematode assemblage composition and distribution patterns (Giere 1993; Heip et al. 1985; Moens et al. 2005). In light of these facts, nematode assemblages are widely regarded as ideal organisms and good indicators of natural and anthropogenic disturbances and changes of environmental conditions in aquatic ecosystems. Furthermore, several studies have highlighted the importance of the link between nematode diversity and ecosystem functioning (Coull & Chandler 1992; Schratzberger et al. 2004; Steyaert et al. 2007; Danovaro et al. 2008; Moreno et al. 2008; Fonseca et al. 2011).

Seagrass beds are characteristic ecosystems of intertidal and shallow subtidal coastal systems in temperate and tropical regions worldwide (Orth et al. 2006), acting as ecosystems engineers by structuring pelagic and benthic assemblages (Bos et al. 2007). They are important marine foundation species, they provide habitat for ecological communities or ecosystems and enhance biodiversity through their facilitative effects on associated species (Ellison et al. 2005). These seagrass beds are important in primary production, nutrient cycling, sediment and nutrient trapping, sediment stabilization, and their structural complexity is critical for the animals which live in them (Boström & Bonsdorff 1997; Orth et al. 2006). As for the benthos, the presence of seagrass reduces physical stress modifying the hydrodynamic environment by stabilizing the sediment, protects smaller invertebrates and enhances food availability (Boström & Bonsdorff 1997). Sediments in seagrass beds typically harbour higher biomass, abundance, diversity and productivity of benthic organisms than unvegetated sediments (Boström et al. 2006). Several studies that analysed the meiobenthic communities associated with sediment seagrass beds have concluded that meiofauna is more abundant and diverse than in bare sediments (Castel et al. 1989; Guerrini et al. 1998; Ndaro & Olafsson 1999; Fisher & Sheaves 2003).

There have been numerous reports of seagrass decline around the world indicating that seagrass habitats are undergoing a global crisis threatening associated organisms (Hughes et al. 2009) and with important consequences to the coastal biodiversity, environmental status and economy (Boström et al. 2006; Hughes et al. 2009; Valle et al. 2014). Although, natural disturbances are recognized, most declines are attributed to anthropogenic disturbances (Short & Wyllie-Echeverria 1996). Their high sensitivity to environmental deterioration and the geographical widespread distribution of these plants

also make seagrasses useful “miner’s canaries” of coastal deterioration (Orth et al. 2006; Marbà et al. 2006).

In the Portuguese coast, seagrass populations are also facing unprecedented declines in distribution, matching the general trends described for most world seagrasses (Cunha et al. 2013). During 2008 the stable *Zostera noltii* (Hornem) seagrass beds in the Mira estuary disappeared completely, leaving behind a muddy area (Adão personal communication; Cunha et al. 2013). This estuary together with its surrounding area, the Mira River, is included in a protected area and it is only subjected to slight human-induced pressures and is considered relatively undisturbed (Costa et al. 2001; Adao et al. 2009). The causes of the habitat loss have not yet been investigated. However, in the last decade important changes in sedimentation dynamics were clearly observed (Adão personal communication). These may result in large-scale alteration of seagrass habitat and have been identified as major drivers of loss (Fourqurean & Rutten 2004) .

During 2009, *Z. noltii* began presenting slight symptoms of natural recovery characterised by pulses with a spatial and temporal irregular distribution of small-sized seagrass patches which changes in habitat configuration. Therefore the horizontal spatial and temporal distribution of seagrass became strongly heterogeneous (Adão personal communication; Cunha et al. 2013) and not homogeneous as expected by habitat loss condition (Elliott et al. 2007).

Most of the restoration and recovery studies in estuarine and coastal water have focused on benthic invertebrates as they are sedentary components closely associated with the sediment. The basic understanding of structure and dynamics of these taxonomic assemblages can provide critical information for the study of these marine systems (Verdonschot et al. 2012). Benthic nematodes recovery is faster (several months) than macrobenthic organisms which is a strong advantage in analysing the structural and

functional assemblage responses during the recovery processes (Borja et al. 2010). The context of the seagrass beds of the Mira estuary creates the natural environmental conditions to examine the fundamental ecology of nematodes assemblages during the early natural recovery processes of seagrass beds from natural induced changes, which implies a passive, ongoing process, depending on a habitat's potential for recovery (Elliott et al. 2007).

Marine nematodes studies in Portugal are scarce, although a comprehensive and reference dataset is available for free living marine nematodes assemblages of the seagrass beds of *Z. noltii* in Mira estuary. This former data correspond to a stable period of this seagrass habitat and it is possible to be identified as a pre-existing ecological condition or state (Borja et al. 2010) before the vanishing of the vegetation. Indeed, this estuary has been used for the generation of a rare database based on the temporal and spatial biodiversity patterns of benthic nematodes (Adão 2004; Adao et al. 2009; Alves et al. 2009).

The present study compares former data of temporal and spatial variability nematodes assemblages in the sediments of the seagrass beds of *Z. noltii* of Mira estuary with the new data collected during the early recovery process. Based on this former and new data it was assessed the effect of the early recovery of seagrass characterised by spatial and temporal alternation of plant patches in diversity, abundance and trophic composition nematode assemblages.

Benthic organisms are generally influenced by complex and interacting physical and biological processes, leading to variation in their distribution at different spatial and temporal scales. If individuals or species interact, or if their environment is not homogeneous, their distribution will have some imprint of this. As such, identifying temporal and spatial distribution patterns is an essential step towards understanding the

processes structuring ecological communities (Underwood & Chapman 1996; Gallucci et al. 2009).

This study aimed to investigate changes in patterns of nematode assemblage composition and biodiversity, trophic composition and life strategies between different environmental conditions of the seagrass habitat: "Before" the habitat loss in stable condition, and "After", during the early recovery of seagrass beds, through the analysis of: *i*) temporal and spatial distribution patterns of nematode communities in both ecological conditions; *ii*) the most important natural environmental variables influencing the nematodes assemblages. The following null hypotheses were tested: *i*) there would be no differences in nematode assemblage composition and biodiversity, density and trophic composition during both environmental conditions, "before" and "after"; and *ii*) there would be no differences in nematode assemblage composition, density and trophic composition at different sampling occasions on both environmental conditions.

Materials and methods

Sampling area and design

Sampling was performed in the Mira estuary, south-western coast of Portugal (37°40'N, 8°40'W), a small mesotidal system with a semidiurnal tidal regime (amplitude 1-3 m during neap and spring tides, respectively). The estuary has a single channel, 5–10 m deep and up to 400 m wide, which allows tidal influence to extend 40 km upstream. Due to the low, seasonal and limited freshwater input, the lower section of the estuary has a dominant marine signature characterised, until 2008, by extensive and homogenous *Z. noltii* meadows, with a strong seasonality with higher biomass in

warmer months (Cunha et al. 2013). Together with its surrounding area, the Mira estuary is included in a protected area, the Natural Park of ‘‘Sudoeste Alentejano e Costa Vicentina’’. This estuary is considered relatively undisturbed and free from major anthropogenic pressures (Costa et al. 2001). The physical and chemical fluctuations mainly result from natural pressures due to its morphology. Upstream tidal penetration is generally limited and determined by the region’s annual rainfall distribution (concentrated between January and March with the rest of the year being usually dry (Paula et al. 2006) and by changes in sedimentation dynamics (Adão personal communication). In 2008, *Z. noltii* meadows disappeared completely. Indications of a natural recovery have been observed since 2009 (Cunha et al. 2013; Materatski et al. in prep., Chapter I).

To compare the temporal and spatial distribution patterns of nematode communities corresponding to stable ecological status and during the early recovery, ‘‘Before’’ and ‘‘After’’ habitat loss, all samples were collected at two sampling sites located in the intertidal sediments of the *Z. noltii* beds; site A, *ca.* 1,5 km from the mouth of the estuary, and site B, 2 km upstream (Fig. 1). Sampling collections were carried out at neap low tide, on five sampling occasions. The former data was sampled in June 1994, September 1994, December 1994, February 1995 and June 1995, at each site two replicates were taken fortnightly, a total of six replicates were analysed at each sampling occasion, except in June 94 when only 4 replicates were studied. During the early recovery period, samples were obtained from the same location and in the similar five sampling occasions, February 2010, June 2010, September 2010, December 2010. At each site there were taken three replicates.

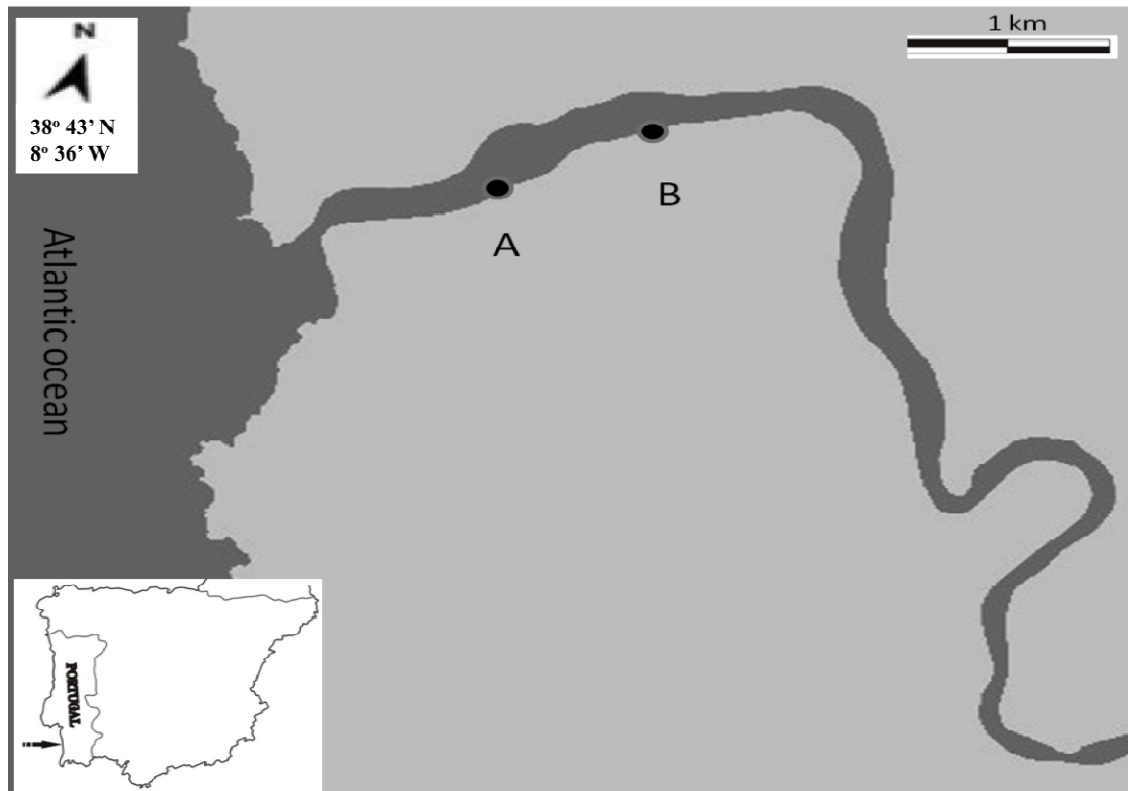


Figure 1. Mira estuary (Portugal): indication of sampling sites (black circles) - (A, *ca.* 1.5 km from the mouth of the estuary, and B, 2 km upstream).

Sampling and sample treatment

Biological Data

Nematode samples of the former data (before) were obtained at each sampling site, by forcing hand corers (3,18 cm inner diameter), to a depth of 3 cm, and during the early recovery of the seagrass (after) the replicate sediment samples of the upper 3 cm were also collected using hand corers (4,6 cm inner diameter). All samples were preserved in a 4% buffered formalin solution. Nematodes were extracted from the sediment using a density gradient centrifugation in colloidal silica (Heip et al. 1985). The fixed samples were rinsed on a 1000 μm mesh sieve followed by sieving on a 38 μm mesh. The fraction retained on the 38 μm sieve was washed and centrifuged three times using the

colloidal silica polymer LUDOX HS-40 (specific gravity 1.19). The supernatant of each washing cycle was again collected on a 38 μm sieve. After extraction all nematodes were counted under a stereomicroscope (40 \times magnification). A random set of 120 nematodes were picked from each replicate, transferred through a graded series of glycerol–ethanol solutions, stored in anhydrous glycerol, and mounted on slides (Vincx 1996). Nematodes were identified to genus level using pictorial keys (Platt & Warwick 1988) and the online identification keys/literature available in the Nemys database (Vanaverbeke et al. 2014). Nematode genus level is considered a taxonomic level with good resolution to discriminate disturbance effects (Warwick et al. 1990; Moreno et al. 2008; Schratzberger et al. 2008).

Environmental data - before vs After

Salinity, temperature ($^{\circ}\text{C}$), pH and dissolved oxygen (DO) (mg L^{-1}) of the overlying water above sediment were measured *in situ*, using different instruments. Before the collapse measurements were done with a salinometer Y.S. I model and WTW 96 probes and after the collapse it was used a WTW InoLab Multi 720 field probe. Additionally, at each site and sampling occasions, water samples of the water column were collected and measured for N and P nutrients ($\mu\text{mol L}^{-1}$) and chlorophyll *a* (mgm^{-3}) in laboratory: nitrate ($\text{NO}_3^{-}\text{-N}$) and nitrite ($\text{NO}_2^{-}\text{-N}$) concentrations were analysed according to standard methods described in Strickland and Parsons (1972) and ammonium ($\text{NH}_4^{+}\text{-N}$) and phosphate ($\text{PO}_4^{3-}\text{-P}$) concentrations were analysed following the Limnologisk Metodik (1992). Chlorophyll *a* (Chl *a*) determinations were performed according to Parsons et al. (1985). At each site and sampling occasion, sediment samples were taken randomly to determine the organic matter content (OM) and grain size. Sediment organic matter content was determined based on the difference between the dry weight

of each sample after oven-drying at 60 °C for 72 h and the weight obtained after combustion at 450 °C for 8 h, and was expressed as the percentage of the total weight. Grain size of the sediments collected before the collapse (former data) was analysed with an automatic C.A. Coulter^R LS Particle Size Analyzer. Grain size of the sediments recently collected was analysed by dry mechanical separation through a column of sieves of different mesh sizes. Based on both methods, the following size frequency distribution of the sediments was determined: the amount of clay (< 4 µm), the amount of silt (between 4 - 63µm) and the amount of sand (>63µm). The relative content of the different grain size fractions was expressed as a percentage.

Zostera noltii was sampled randomly at each site and sampling occasion, three replicate samples were taken at each site before the collapse and three replicate samples were taken after, in both periods of time sediment hand-corers with a surface area of 141 cm⁻² and 30 cm in depth, were used. On each replicate, the roots were separated from the leaves and then dried in an oven at 60 °C for 48 hours. The leaves and the root biomass was estimated by the organic weight and the ash-free dry weight (gm⁻² AFDW—ash free dry weight). Ash-free dry weight was obtained as the weigh loss of the dry material after combustion at 450 °C for 8 hours, in a muffle furnace (Heraeus KR 170E).

Data Analysis

Univariate and multivariate analyses were performed to detect temporal and spatial changes in the community structure between “Sites” and “Sampling occasions” under two ecological conditions: stable ecological status “Before” and “After” habitat loss, during the early recovery of the seagrass beds. The statistical analysis was performed using the PRIMER v6 software package (Clarke & Warwick 2001) with the PERMANOVA add-on package (Anderson et al. 2008).

Environmental variables - before vs after

A Principal Component Analysis (PCA) of the environmental variables measured was performed to find patterns in multidimensional data by reducing the number of dimensions, with minimal loss of information. The PCA ordination was based on the average of the environmental factors measured “Before” and “After “ the habitat loss, by “Sites” and “Sampling occasions”. Prior to the calculation of the environmental parameter resemblance matrix based on Euclidean distances, data were $\log(X+1)$ transformed followed normalization. Selective transformations were required for the water environmental variables, Chlorophyll *a*, nitrate, nitrite, ammonium and phosphate concentrations of the water and sediments, to follow the assumptions for calculating normalized Euclidean distances.

Nematode assemblages - before vs after

Total nematode density (individuals 10 cm⁻²), genera composition and diversity, trophic composition and several ecological indicators, either based on diversity (Margalef Index, *d* and Shannon-Wiener diversity, *H'*) or on ecological strategies (Index of Trophic Diversity, *ITD*; Maturity Index , *MI*), were calculated using the nematodes dataset, for Before and After the habitat loss, each site and sampling occasion. In order to investigate the trophic composition of the assemblages, nematodes genera were assigned to one of four feeding groups, designated by Wieser (1953), mainly on the basis of the mouth morphology, including presence or absence of prominent buccal armature. Based on the feeding-type classification from Wieser (1953), the Index of Trophic Diversity (*ITD*) was calculated (Heip et al. 1985). The reciprocal value of the

trophic index (θ') was used, so that the higher values of the index correspond to higher trophic diversity.

The Maturity Index (Bongers 1990; Bongers et al. 1991) was used to analyse nematodes' life strategy. Nematode genera identified were assigned a value on a colonizer–persister scale (*c–p scale*) from 2 (colonizers) to 5 (persisters), where taxa with rapid growth and reproduction and usually high tolerance to disturbance are considered colonizers, whereas persisters are slow-growing and often more sensitive taxa which thrive well in fairly stable and pristine environments (Bongers 1990; Bongers et al. 1991). Thus, the *c–p* scores reflect life-history characteristics associated with r- and K-selection for colonizers and persisters, respectively (Bongers & Bongers 1998; Bongers & Ferris 1999). The maturity index is calculated as the weighted average of the individual colonizer–persister (*c–p*) scores as $MI = \sum_{i=1}^n v(i) \times f(i)$ where $v(i)$ is the *c–p* value of the taxon i and $f(i)$ is the frequency of that taxon. Based on this classification, nematode genera collected in both periods of sampling were assigned *c–p* scores ranging from 2 to 4.

A two-way permutational analysis of variance (PERMANOVA) was applied to test the null hypothesis suggesting that no significant temporal differences (between Before-After habitat loss and between Sampling occasions) and spatial (between sites) existed in nematode assemblages descriptors: total density, genera composition and diversity, trophic composition, d , H' , ITD, and MI. The PERMANOVA analysis was carried out following the three factor design: Time “Before” and “After” (2 levels, fixed); “Site” A and B (2 levels, random) and “Sampling occasion” June (1994, 1995 and 2010), September (1994 and 2010), December (1994 and 2010), February (1995, 2010 and 2011) (10 levels, random nested in time). The PERMANOVA analysis was also performed to test the null hypothesis suggesting that no significant differences between

sampling occasions existed before and after collapse, with two factors design: “Site” A and “Site” B (2 levels, fixed) and “Sampling occasion” (10 levels, fixed).

Nematode density data were square root transformed in order to scale down densities of highly abundant nematode species and therefore increase the importance of the less abundant in analysis and similarity between communities before and after habitat loss, in the different sites and sampling occasions. The PERMANOVA analysis was conducted on a Bray-Curtis similarity matrix (Clarke & Green 1988). The null hypothesis was rejected at a significance level <0.05 (if the number of permutations was lower than 150, the Monte Carlo permutation p was used). Whenever significant interactions in effects of the factors were detected, these were examined using *a posteriori* pairwise comparisons, using 9999 permutations under a reduced model. The similarity in communities between before and after, sites and sampling occasions were plotted by Principal coordinates analysis (PCO) using the Bray-Curtis similarity measure.

The relative contribution of each genus to the average dissimilarities between time, sites and sampling occasions was calculated using two way-crossed similarity percentage analysis (SIMPER, cut-off percentage: 90%).

The relationship between environmental variables and the structure of the nematode community was explored by carrying out the BIOENV procedure (Clarke & Ainsworth 1993), using Spearman’s correlation.

Results

Environmental variables

Based on the results of the environmental variables measured there were clear differences between the two ecological status, “Before” the habitat loss and “After,” during the early recovery of the seagrass beds. An accentuated decrease in biomass of the *Z. noltii* and OM of sediments was obtained and the grain size of sediments registered an important increase (Table 1).

Table 1. pH, Sal, salinity; T, temperature; O₂, dissolved oxygen; O₂, percentage oxygen; NH₄⁺, ammonium; PO₄³⁻, phosphate; NO₂⁻, nitrite; NO₃⁻, nitrate; Sili, Silicate; Chl-a, chlorophyll a; MO, organic matter; Clay (< 4 µm); Silt (between 4 - 63µm); Sand (>63µm); Leaves (gm⁻² AFDW—ash free dry weight); Roots (gm⁻² AFDW—ash free dry weight).

| Time | Sampling occasions | Sites | pH | Sal | T °C | O ₂ (%) | O ₂ mg/l | NH ₄ ⁺ µmol/L | PO ₄ ³⁻ µmol/L | NO ₂ ⁻ µmol/L | NO ₃ ⁻ µmol/L | Sili µmol/L | Chl-a mg/m ³ | MO (%) | Clay (%) | Silt (%) | Sand (%) | AFDW Leaves g/m ² | AFDW Roots g/m ² |
|--------|--------------------|-------|-----|------|---------|-----------------------|------------------------|--|---|--|--|----------------|----------------------------|-----------|-------------|-------------|-------------|------------------------------------|-----------------------------------|
| Before | Jun 1994 | A | 8.3 | 34.7 | 23.9 | 149.2 | 12.5 | 9.7 | 0.2 | 0.2 | 0.4 | 3.2 | 1.6 | 8.9 | 15.9 | 64.8 | 19.4 | 37.0 | 24.2 |
| | | B | 8.4 | 34.8 | 27.0 | 135.2 | 11.8 | 7.7 | 0.7 | 0.1 | 0.7 | 16.1 | 1.5 | 10.1 | 12.0 | 49.9 | 38.0 | 43.7 | 27.3 |
| | Sep 1994 | A | 8.5 | 33.8 | 18.4 | 128.1 | 11.1 | 7.0 | 0.7 | 0.2 | 1.0 | 5.3 | 1.9 | 8.9 | 18.1 | 59.8 | 22.2 | 18.2 | 36.8 |
| | | B | 8.6 | 33.9 | 19.9 | 168.2 | 15.9 | 17.2 | 0.7 | 0.2 | 0.8 | 11.9 | 5.7 | 9.9 | 13.4 | 57.1 | 29.5 | 23.8 | 28.7 |
| | Dec 1994 | A | 8.6 | 33.3 | 17.8 | 144.8 | 13.7 | 24.9 | 0.5 | 0.3 | 2.4 | 7.3 | 1.1 | 10.2 | 9.5 | 45.4 | 45.2 | 20.8 | 53.3 |
| | | B | 8.5 | 33.7 | 16.5 | 135.8 | 13.6 | 20.3 | 11.4 | 0.2 | 5.8 | 5.3 | 1.6 | 10.5 | 12.0 | 46.9 | 41.1 | 14.2 | 20.5 |
| | Feb 1995 | A | 8.8 | 35.3 | 17.3 | 148.8 | 13.6 | 24.0 | 0.5 | 0.4 | 1.0 | 5.5 | 0.9 | 9.3 | 9.7 | 44.3 | 45.9 | 24.9 | 48.7 |
| | | B | 8.8 | 34.6 | 15.4 | 154.6 | 12.8 | 18.1 | 0.2 | 0.3 | 0.7 | 4.1 | 1.7 | 10.1 | 11.0 | 46.6 | 42.4 | 11.4 | 23.7 |
| | Jun 1995 | A | 8.7 | 36.1 | 25.3 | 169.0 | 13.9 | 15.3 | 0.4 | 0.3 | 0.7 | 7.4 | 2.6 | 10.6 | 14.1 | 54.5 | 31.4 | 37.0 | 47.0 |
| | | B | 8.7 | 36.2 | 26.0 | 159.0 | 12.4 | 22.6 | 0.5 | 0.2 | 2.6 | 2.7 | 5.0 | 10.4 | 12.8 | 52.9 | 34.3 | 33.5 | 35.1 |
| After | Feb 2010 | A | 8.1 | 28.0 | 19.0 | 47.6 | 5.1 | 3.9 | 0.4 | 0.7 | 4.7 | 0.5 | 0.9 | 5.4 | 0.7 | 8.7 | 90.6 | 0.1 | 1.5 |
| | | B | 8.2 | 27.8 | 17.0 | 47.8 | 5.1 | 6.7 | 0.3 | 0.8 | 2.2 | 0.3 | 0.1 | 5.0 | 0.2 | 5.9 | 93.8 | 1.8 | 7.7 |
| | Jun 2010 | A | 8.1 | 35.0 | 21.5 | 110.0 | 9.7 | 10.8 | 0.1 | 0.4 | 1.0 | 0.2 | 2.5 | 5.1 | 3.2 | 15.6 | 81.2 | 1.0 | 6.6 |
| | | B | 8.2 | 35.1 | 23.7 | 111.5 | 10.3 | 10.5 | 0.1 | 0.1 | 0.2 | 0.2 | 4.9 | 3.7 | 2.2 | 15.1 | 82.6 | 0.0 | 0.0 |
| | Sep 2010 | A | 8.1 | 35.0 | 21.5 | 110.0 | 9.7 | 6.6 | 0.3 | 0.1 | 0.3 | 0.1 | 1.8 | 6.6 | 6.1 | 13.9 | 80.7 | 2.3 | 4.8 |
| | | B | 8.2 | 35.1 | 23.7 | 111.5 | 10.3 | 3.4 | 0.2 | 1.0 | 1.1 | 0.1 | 3.1 | 6.3 | 6.9 | 12.1 | 81.2 | 0.4 | 1.7 |
| | Dec 2010 | A | 7.9 | 34.6 | 23.0 | 20.0 | 1.8 | 4.7 | 0.2 | 1.0 | 7.3 | 0.4 | 1.2 | 6.4 | 5.2 | 16.0 | 78.8 | 0.1 | 0.0 |
| | | B | 7.8 | 33.9 | 22.1 | 18.3 | 1.6 | 19.7 | 0.2 | 0.1 | 3.6 | 0.2 | 3.5 | 6.1 | 2.8 | 12.7 | 84.5 | 0.3 | 2.5 |
| | Feb 2011 | A | 8.3 | 25.7 | 17.3 | 45.2 | 4.4 | 10.0 | 0.4 | 0.6 | 4.0 | 10.8 | 0.9 | 2.4 | 5.0 | 2.1 | 92.9 | 0.2 | 1.5 |
| | | B | 8.2 | 23.5 | 15.4 | 41.1 | 4.2 | 11.0 | 0.3 | 0.7 | 3.9 | 4.1 | 0.1 | 7.5 | 5.1 | 2.9 | 91.9 | 2.0 | 6.9 |

The PCA ordination of the environmental factors showed that the first two components (PC1, 52.7% and PC2, 15.6%) accounted for about 68.2% of the variability of data (Fig. 2). The PCA ordination separated samples collected before the vanishing of the seagrass bed from the remaining sampled during the early recovery process of the ecosystem, mainly due to the highest values of *Z. noltii* biomass, highest values of the nutrients water concentrations, chlorophyll *a* and temperature. The samples from early

recovery status were characterised by a higher percentage of sand and highest values of the water oxygen concentration, pH, water silicates concentration and lower organic matter sediment values.

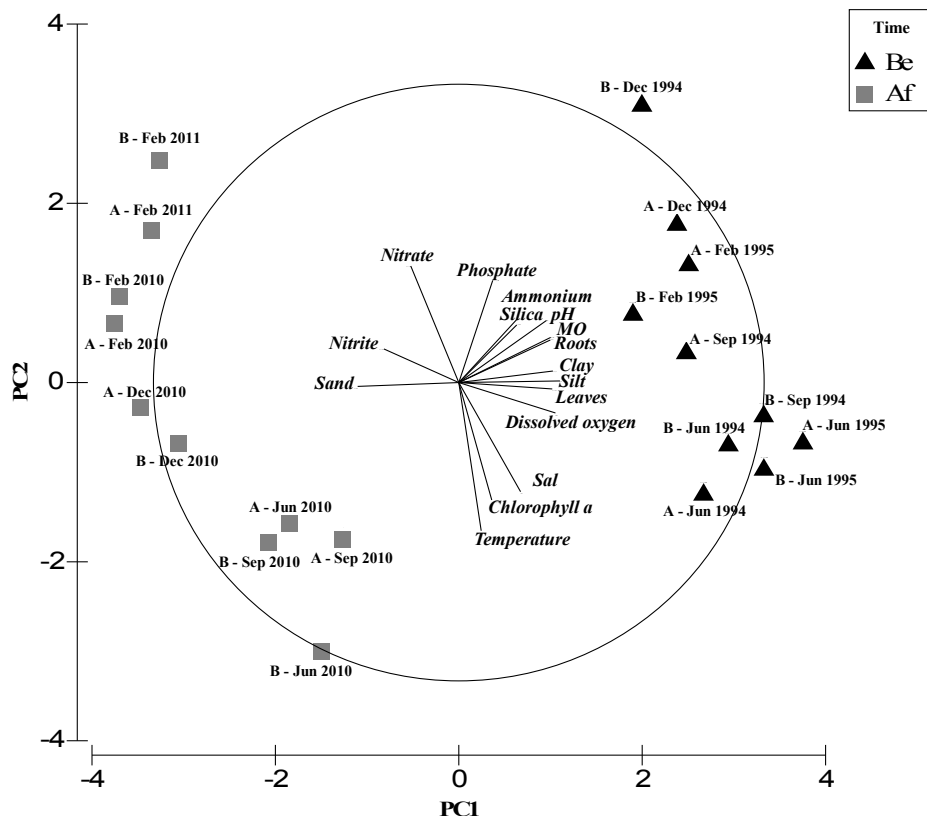


Figure 2. Principal Component Analysis (PCA) plot based on the environmental variables measured in each “Time” before and after (2 levels, fixed), “Site” A and B (2 levels, random) and “Sampling Occasion” June (1994, 1995 and 2010), September (1994 and 2010), December (1994 and 2010), February (1995, 2010 and 2011) (10 levels, random nested in “Time”). PC1 = 52.7%, PC2 = 15.6%.

Before the collapse, the biomass of *Z. noltii* was deeply higher and the temporal variation of biomass of *Z. noltii* followed clearly seasonal patterns, characterised by maximum values of the leaves biomass in the summer (Site A, June 1994, 1995- 37.0

gm⁻²; Site B June 1994- 43.7 gm⁻², June 1995, 33.5 gm⁻²) and minimum values in the winter (Site A, December 1994- 20.8 gm⁻²; Site B, December 1994, 14.2 gm⁻² February 1995, 11.4 gm⁻²). As expected, during the early recovery the *Z. noltii* the biomass was very low and registered strong fluctuations throughout the study period, ranging from the complete absence of leaves biomass in Site B in June 2010 and Site A in December 2010, to the maximum values obtained at Site A in September 2010 and at Site B in February 2011 both presenting 2.3 gm⁻².

In general a temporal pattern of the environmental variables was not observed before the collapse of the *Z. noltii*, though some temporal patterns of the *Z. noltii* biomass were detected. During the recovery period, a temporal trend was clearly observed in the ordination. The samples of June 2010 and September 2010 were separated from those of December 2010 and February 2011, due to high values of chlorophyll *a* in June 2010 and high salinity in September 2010.

Nematodes assemblages - density

In all sampling occasions the density of nematodes was consistently higher before the habitat loss, corresponding to stable condition (Table 2). Significant differences were obtained between “Before and After” (factor Time, $p < 0.05$) as well as between sites (factor “Site”, $p < 0.05$) (Table 3). Before the collapse of the seagrass, at site A the mean density (\pm SE) was 1798 ± 180 ind. 10 cm⁻² and ranged from 644 ± 115 ind. 10 cm⁻² (June 95) to 2628 ± 448 ind. 10 cm⁻² (February 95). At site B, the mean density (\pm SE) was 3338 ± 517 ind. 10 cm⁻² and ranged from 1276 ± 279 ind. 10 cm⁻² (June 95) to 6242 ± 1344 ind. 10 cm⁻² (February 95). After the collapse the density of nematodes was lower, at site A the mean density (\pm SE) was 1119 ± 147 ind. 10 cm⁻² and ranged from 705 ± 86 ind. 10 cm⁻² (February 11) to 1615 ± 119 ind. 10 cm⁻² (September 10). At

site B, the mean density was 2819 ± 406 ind. 10 cm^{-2} and ranged from 826 ± 169 (February 11) ind. 10 cm^{-2} to 3533 ± 182 ind. 10 cm^{-2} (February 10) (Fig. 3). Moreover, nematode density results showed significant differences between sampling occasions (factor “Sampling occasions”, $p > 0.05$) (Table 3).

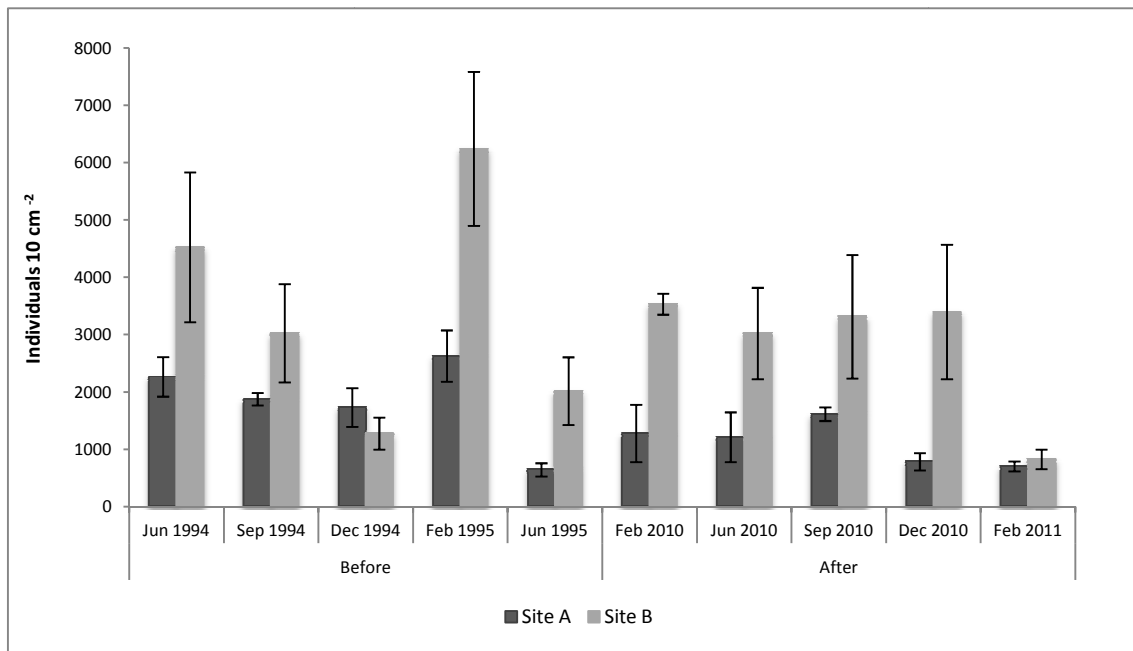


Figure 3. Nematode community density (ind 10 cm^{-2}) average values and standard error (\pm SE) in each sampling occasion: June (1994, 1995 and 2010); September (1994 and 2010); December (1994 and 2010); February (1995, 2010 and 2011) and distribution across the sites (A and B).

Table 2. The most abundant nematode genera (individuals 10 cm⁻²) before and after the collapse of *Z. noltii*, average density and standard error (\pm SE) in each sampling occasion; June (1994, 1995 and 2010), September (1994 and 2010), December (1994 and 2010), February (1995, 2010 and 2011) and distribution across the sites (A and B). Only the most abundant genera are included in this table.

| Genera | Before | | | | | | | | | | After | | | | | | | | | |
|------------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| | Site A | | | | | Site B | | | | | Site A | | | | | Site B | | | | |
| | Jun 1994 \pm SE | Sep 1994 \pm SE | Dec 1994 \pm SE | Feb 1995 \pm SE | Jun 1995 \pm SE | Jun 1994 \pm SE | Sep 1994 \pm SE | Dec 1994 \pm SE | Feb 1995 \pm SE | Jun 1995 \pm SE | Feb 2010 \pm SE | Jun 2010 \pm SE | Sep 2010 \pm SE | Dec 2010 \pm SE | Feb 2011 \pm SE | Feb 2010 \pm SE | Jun 2010 \pm SE | Sep 2010 \pm SE | Dec 2010 \pm SE | Feb 2011 \pm SE |
| <i>Terschellingia</i> | 638 \pm 145 | 647 \pm 177 | 274 \pm 69 | 617 \pm 141 | 58 \pm 28 | 1031 \pm 278 | 1074 \pm 613 | 253 \pm 102 | 2162 \pm 765 | 229 \pm 87 | 180 \pm 110 | 247 \pm 184 | 280 \pm 68 | 125 \pm 25 | 71 \pm 26 | 531 \pm 225 | 728 \pm 222 | 585 \pm 419 | 948 \pm 409 | 157 \pm 45 |
| <i>Paracomescoma</i> | 590 \pm 209 | 494 \pm 94 | 223 \pm 52 | 643 \pm 154 | 88 \pm 71 | 456 \pm 129 | 338 \pm 139 | 180 \pm 72 | 968 \pm 221 | 65 \pm 36 | 64 \pm 27 | 29 \pm 11 | 453 \pm 26 | 42 \pm 16 | 81 \pm 16 | 662 \pm 54 | 336 \pm 186 | 753 \pm 244 | 350 \pm 173 | 103 \pm 17 |
| <i>Spirinia</i> | 29 \pm 5 | 90 \pm 20 | 205 \pm 46 | 158 \pm 39 | 268 \pm 106 | 482 \pm 150 | 767 \pm 162 | 262 \pm 118 | 348 \pm 215 | 966 \pm 430 | 269 \pm 88 | 50 \pm 1 | 316 \pm 109 | 122 \pm 23 | 64 \pm 13 | 195 \pm 79 | 82 \pm 82 | 13 \pm 13 | 35 \pm 17 | 31 \pm 28 |
| <i>Odonthophora</i> | 534 \pm 181 | 198 \pm 41 | 159 \pm 55 | 385 \pm 59 | 59 \pm 20 | 547 \pm 95 | 221 \pm 39 | 119 \pm 43 | 540 \pm 87 | 191 \pm 96 | 98 \pm 23 | 239 \pm 196 | 220 \pm 35 | 69 \pm 31 | 92 \pm 14 | 274 \pm 54 | 210 \pm 82 | 217 \pm 77 | 243 \pm 41 | 18 \pm 4 |
| <i>Linhomeus</i> | 117 \pm 51 | 67 \pm 27 | 116 \pm 37 | 133 \pm 30 | 17 \pm 6 | 350 \pm 110 | 196 \pm 58 | 86 \pm 26 | 696 \pm 268 | 95 \pm 33 | 31 \pm 9 | 89 \pm 27 | 50 \pm 26 | 27 \pm 1 | 13 \pm 3 | 119 \pm 44 | 134 \pm 82 | 342 \pm 231 | 145 \pm 86 | 54 \pm 18 |
| <i>Daptonema</i> | 117 \pm 41 | 18 \pm 10 | 38 \pm 14 | 162 \pm 27 | 22 \pm 11 | 141 \pm 46 | 15 \pm 8 | 36 \pm 14 | 235 \pm 55 | 141 \pm 75 | 47 \pm 14 | 20 \pm 7 | 34 \pm 2 | 67 \pm 21 | 70 \pm 12 | 315 \pm 127 | 113 \pm 16 | 98 \pm 39 | 82 \pm 23 | 85 \pm 36 |
| <i>Metalinhomeus</i> | 3 \pm 3 | 30 \pm 27 | 0 | 6 \pm 6 | 2 \pm 2 | 0 | 0 | 0 | 0 | 0 | 182 \pm 80 | 58 \pm 50 | 62 \pm 26 | 58 \pm 21 | 7 \pm 7 | 46 \pm 46 | 0 | 80 \pm 40 | 111 \pm 81 | 22 \pm 22 |
| <i>Metachromadora</i> | 57 \pm 22 | 16 \pm 9 | 5 \pm 5 | 22 \pm 19 | 0 | 293 \pm 112 | 22 \pm 14 | 3 \pm 2 | 152 \pm 64 | 12 \pm 5 | 1 \pm 1 | 16 \pm 9 | 9 \pm 9 | 5 \pm 5 | 0 | 180 \pm 180 | 93 \pm 29 | 329 \pm 127 | 291 \pm 137 | 21 \pm 13 |
| <i>Paracytholaimus</i> | 0 | 20 \pm 8 | 40 \pm 15 | 143 \pm 41 | 2 \pm 2 | 12 \pm 8 | 26 \pm 20 | 34 \pm 6 | 25 \pm 11 | 10 \pm 5 | 19 \pm 12 | 2 \pm 2 | 0 | 29 \pm 18 | 18 \pm 5 | 371 \pm 154 | 33 \pm 25 | 30 \pm 25 | 58 \pm 21 | 33 \pm 18 |
| <i>Sphaerolaimus</i> | 3 \pm 3 | 49 \pm 27 | 9 \pm 9 | 3 \pm 3 | 0 | 0 | 0 | 5 \pm 3 | 84 \pm 46 | 5 \pm 5 | 38 \pm 23 | 66 \pm 20 | 20 \pm 5 | 14 \pm 4 | 47 \pm 6 | 42 \pm 28 | 74 \pm 29 | 109 \pm 98 | 86 \pm 26 | 32 \pm 12 |
| <i>Axonolaimus</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 39 \pm 16 | 89 \pm 17 | 30 \pm 10 | 10 \pm 4 | 40 \pm 15 | 53 \pm 21 | 34 \pm 19 | 76 \pm 25 | 46 \pm 10 | 35 \pm 12 |
| <i>Chromadorella</i> | 0 | 49 \pm 20 | 250 \pm 125 | 50 \pm 13 | 17 \pm 7 | 0 | 30 \pm 11 | 22 \pm 9 | 75 \pm 27 | 6 \pm 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>Viscosia</i> | 34 \pm 26 | 14 \pm 11 | 41 \pm 15 | 36 \pm 15 | 19 \pm 9 | 65 \pm 39 | 37 \pm 12 | 32 \pm 10 | 65 \pm 21 | 52 \pm 16 | 51 \pm 41 | 13 \pm 13 | 0 | 11 \pm 3 | 23 \pm 13 | 21 \pm 11 | 0 | 59 \pm 19 | 34 \pm 8 | 7 \pm 7 |
| <i>Dichromadora</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 46 \pm 25 | 0 | 0 | 0 | 0 | 147 \pm 114 | 0 | 18 \pm 8 | 3 \pm 3 |
| <i>Ptycholaimellus</i> | 7 \pm 7 | 6 \pm 4 | 5 \pm 3 | 18 \pm 12 | 12 \pm 6 | 23 \pm 23 | 10 \pm 10 | 11 \pm 7 | 97 \pm 39 | 36 \pm 20 | 22 \pm 19 | 57 \pm 11 | 14 \pm 9 | 65 \pm 25 | 73 \pm 16 | 194 \pm 65 | 362 \pm 235 | 190 \pm 88 | 208 \pm 101 | 18 \pm 7 |
| <i>Sabatieria</i> | 0 | 0 | 3 \pm 3 | 0 | 0 | 0 | 0 | 0 | 0 | 4 \pm 4 | 7 \pm 6 | 26 \pm 13 | 11 \pm 11 | 17 \pm 9 | 9 \pm 6 | 56 \pm 30 | 168 \pm 79 | 151 \pm 45 | 479 \pm 246 | 112 \pm 20 |
| <i>Atrochromadora</i> | 5 \pm 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 72 \pm 31 | 41 \pm 31 | 19 \pm 13 | 5 \pm 2 | 4 \pm 2 | 75 \pm 14 | 149 \pm 72 | 0 | 41 \pm 30 | 14 \pm 4 |
| <i>Eleutherolaimus</i> | 11 \pm 11 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 \pm 1 | 14 \pm 14 | 0 | 24 \pm 24 | 2 \pm 2 | 44 \pm 31 | 34 \pm 25 | 74 \pm 4 | 42 \pm 7 | 10 \pm 5 |
| <i>Oncholaimellus</i> | 0 | 10 \pm 6 | 40 \pm 31 | 4 \pm 3 | 4 \pm 3 | 5 \pm 5 | 6 \pm 6 | 18 \pm 15 | 36 \pm 36 | 14 \pm 14 | 34 \pm 16 | 33 \pm 4 | 10 \pm 5 | 15 \pm 5 | 33 \pm 7 | 21 \pm 21 | 62 \pm 25 | 4 \pm 4 | 21 \pm 15 | 2 \pm 2 |
| <i>Desmodora</i> | 0 | 0 | 0 | 0 | 1 \pm 1 | 421 \pm 256 | 104 \pm 104 | 2 \pm 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>Molgolaimus</i> | 3 \pm 3 | 3 \pm 3 | 10 \pm 5 | 20 \pm 9 | 0 | 202 \pm 114 | 6 \pm 6 | 17 \pm 14 | 186 \pm 72 | 7 \pm 7 | 0 | 0 | 0 | 0 | 0 | 0 | 5 \pm 5 | 0 | 0 | 0 |
| <i>Chromadorina</i> | 7 \pm 7 | 5 \pm 5 | 97 \pm 76 | 42 \pm 22 | 1 \pm 1 | 0 | 43 \pm 30 | 71 \pm 37 | 77 \pm 42 | 4 \pm 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 \pm 4 | 0 | 0 |
| Other genera | 101 \pm 96 | 157 \pm 141 | 216 \pm 145 | 185 \pm 113 | 73 \pm 60 | 496 \pm 278 | 131 \pm 94 | 126 \pm 88 | 496 \pm 435 | 181 \pm 119 | 122 \pm 92 | 79 \pm 40 | 88 \pm 54 | 77 \pm 41 | 58 \pm 45 | 334 \pm 220 | 258 \pm 207 | 202 \pm 188 | 160 \pm 129 | 69 \pm 36 |

Table 3. Details of the three-factor PERMANOVA test in each “Time” before and after (2 levels, fixed), “Site” A and B (2 levels, random) and “Sampling Occasion” June (1994, 1995 and 2010), September (1994 and 2010), December (1994 and 2010), February (1995, 2010 and 2011) (10 levels, random nested in “Time”), for all variables analysed. Bold values stand for the significant differences ($p < 0.05$).

| Source of variation | | Degrees of freedom | Sum of squares | Mean squares | Pseudo-F | perms | P(perm) |
|----------------------------|---------------------------------|--------------------|----------------|--------------|----------|-------|---------------|
| Nematode total density | Time | 1 | 22348 | 22348 | 3.2186 | 9949 | 0.0365 |
| | Site | 1 | 6691 | 6691 | 5.1211 | 9920 | 0.0051 |
| | Sampling Occasion (Time) | 8 | 27594 | 3449.2 | 2.5503 | 9891 | 0.0002 |
| | Time x Site | 1 | 4207.6 | 4207.6 | 3.2203 | 9928 | 0.0115 |
| | Site x Sampling Occasion (Time) | 8 | 10820 | 1352.5 | 1.5408 | 9803 | 0.0008 |
| | Residual | 66 | 57935 | 877.8 | | | |
| | Total | 85 | 1.29E+05 | | | | |
| Number of genera | Time | 1 | 1475.7 | 1475.7 | 33.28 | 9962 | 0.0002 |
| | Site | 1 | 35.692 | 35.692 | 1.5893 | 9894 | 0.2377 |
| | Sampling Occasion (Time) | 8 | 360.29 | 45.036 | 1.9691 | 9955 | 0.1816 |
| | Time x Site | 1 | 4.2849 | 4.2849 | 0.19079 | 9911 | 0.6836 |
| | Site x Sampling Occasion (Time) | 8 | 182.97 | 22.871 | 1.2296 | 9944 | 0.2937 |
| | Residual | 66 | 1227.6 | 18.6 | | | |
| | Total | 85 | 3254.8 | | | | |
| Trophic composition | Time | 1 | 1236.2 | 1236.2 | 0.76786 | 9949 | 0.6793 |
| | Site | 1 | 4045 | 4045 | 8.1192 | 9949 | 0.0118 |
| | Sampling Occasion (Time) | 8 | 10821 | 1352.6 | 2.5948 | 9951 | 0.0511 |
| | Time x Site | 1 | 1001.5 | 1001.5 | 2.0101 | 9948 | 0.1439 |
| | Site x Sampling Occasion (Time) | 8 | 4170.2 | 521.28 | 1.8429 | 9910 | 0.0168 |
| | Residual | 66 | 18669 | 282.86 | | | |
| | Total | 85 | 39456 | | | | |
| Margalef index | Time | 1 | 1504.3 | 1504.3 | 22.485 | 9966 | 0.0008 |
| | Site | 1 | 2.8206 | 2.8206 | 0.12325 | 9915 | 0.7764 |
| | Sampling Occasion (Time) | 8 | 466.62 | 58.327 | 2.499 | 9960 | 0.1107 |
| | Time x Site | 1 | 15.155 | 15.155 | 0.66223 | 9918 | 0.435 |
| | Site x Sampling Occasion (Time) | 8 | 186.72 | 23.34 | 1.2524 | 9948 | 0.2828 |
| | Residual | 66 | 1230 | 18.636 | | | |
| | Total | 85 | 3346.2 | | | | |
| Shannon-Wiener index | Time | 1 | 283.99 | 283.99 | 23.839 | 9973 | 0.0004 |
| | Site | 1 | 5.2678 | 5.2678 | 1.0179 | 9882 | 0.341 |
| | Sampling Occasion (Time) | 8 | 94.941 | 11.868 | 2.235 | 9955 | 0.1472 |
| | Time x Site | 1 | 1.3946 | 1.3946 | 0.26949 | 9907 | 0.6233 |
| | Site x Sampling Occasion (Time) | 8 | 42.478 | 5.3098 | 1.3553 | 9936 | 0.2369 |
| | Residual | 66 | 258.57 | 3.9178 | | | |
| | Total | 85 | 681.08 | | | | |
| Index of trophic diversity | Time | 1 | 508.48 | 508.48 | 4.4808 | 9962 | 0.0603 |
| | Site | 1 | 0.24567 | 0.24567 | 6.14E-03 | 9916 | 0.9719 |
| | Sampling Occasion (Time) | 8 | 891.58 | 111.45 | 2.6773 | 9964 | 0.0918 |
| | Time x Site | 1 | 21.209 | 21.209 | 0.53005 | 9906 | 0.4969 |
| | Site x Sampling Occasion (Time) | 8 | 333.02 | 41.627 | 1.6687 | 9946 | 0.1181 |
| | Residual | 66 | 1646.4 | 24.946 | | | |
| | Total | 85 | 3476 | | | | |
| Maturity Index | Time | 1 | 26.041 | 26.041 | 1.4658 | 9955 | 0.3393 |
| | Site | 1 | 0.89717 | 0.89717 | 0.4708 | 9862 | 0.5194 |
| | Sampling Occasion (Time) | 8 | 71.792 | 8.974 | 4.6385 | 9968 | 0.0291 |
| | Time x Site | 1 | 10.853 | 10.853 | 5.6949 | 9875 | 0.0459 |
| | Site x Sampling Occasion (Time) | 8 | 15.477 | 1.9347 | 1.1834 | 9953 | 0.3194 |
| | Residual | 66 | 107.9 | 1.6348 | | | |
| | Total | 85 | 238.25 | | | | |

Nematodes assemblages- Structural diversity

In the former data, nematodes were identified as belonging to 58 genera and 21 families. Most genera belonged to the orders Monhysterida (51.4%), Chromadorida (44.9%) and Enoplida (3.7%); the dominant families were Linhomoeidae (35.0%), Desmodoridae (19.7%), Comesomatidae (16.1%), Axonolaimidae (10.9%). The five genera *Terschellingia* (26.8%), *Paracomesoma* (15.4%), *Spirinia* (14.2%), *Odonthophora* (10.8%) and *Linhomoeus* (7.2%) together comprised nearly 75% of nematode abundances and fifteen genera accounted for 90% of the total nematode density. During the early recovery, after the habitat loss, nematodes were identified as belonging to 50 genera and 22 families. Most genera belonged to the same three orders, Monhysterida (48.2%), Chromadorida (47.1%) and Enoplida (4.7%); the dominant families were Linhomoeidae (29.1%), Comesomatidae (20.6%), Axonolaimidae (10.8%), Desmodoridae (10.8%). The nine genera, *Terschellingia* (19.6%), *Paracomesoma* (14.6%), *Odonthophora* (8.5%), *Ptycholaimellus* (6.1%), *Spirinia* (6.0%), *Sabatieria* (5.3%), *Linhomoeus* (5.1%), *Metachromadora* (4.8%) and *Daptonema* (4.7%) together comprised nearly 75% of nematode abundances and sixteen genera accounted for 90% of the total nematode density.

The number of genera before the collapse ranged, at site A, from 9, in September 1994 to 22, in February 1995. At site B it ranged from 11, in September 1994 and 20, in February 1995. After the collapse the number of genera ranged, at site A, from 13, in September 2010 and 25, in December 2010. At site B it ranged from 16, in September 2010 and 24, in June 2010. PERMANOVA revealed that the number of genera was significant different between “Before and After” (factor Time, $p < 0.05$) (Table 3), with higher values after the collapse of *Z. noltii*.

Species richness and structural diversity, based on Margalef Index (d) and Shannon–Wiener values (H') increased after the collapse, during the recovery process. Before the collapse, d values ranged from 1.36 ± 0.10 in June 1994 at site A to 2.14 ± 0.10 in December 1994 at site B. After the collapse, d values ranged from 1.93 ± 0.14 in September 2010 at site A to 3.31 ± 0.16 in December 2010 at site B (Fig. 4). As for the H' values, before the collapse they ranged from 1.67 ± 0.05 in September 1994 at site B to 2.23 ± 0.05 in December 1994 at site A. During the early recovery H' values ranged from 2.01 ± 0.08 in September 2010 to 2.61 ± 0.06 in December 2010 both at site A (Fig. 4). The PERMANOVA analysis applied to both indices showed significant differences between the factor Time “Before” and “After” (factor “Time”, $p > 0.05$), although it did not show significant differences between sites (factor “Site”, $p > 0.05$) or sampling occasions (factor “Sampling occasions”, $p > 0.05$) (Table 3). The individual pairwise comparisons were performed following the factor design “Site” x “Sampling Occasion”, revealing a low variability among sampling occasions, although significant differences were detected ($p < 0.05$): at site A, between: June (1994, 1995, 2010); December 1994 and 2010; February 1995 and 2010; February 1995 and 2011; at site B, between: June 1994 and 2010; September 1994 and 2010; February 1995 and 2010; February 1995 and 2011.

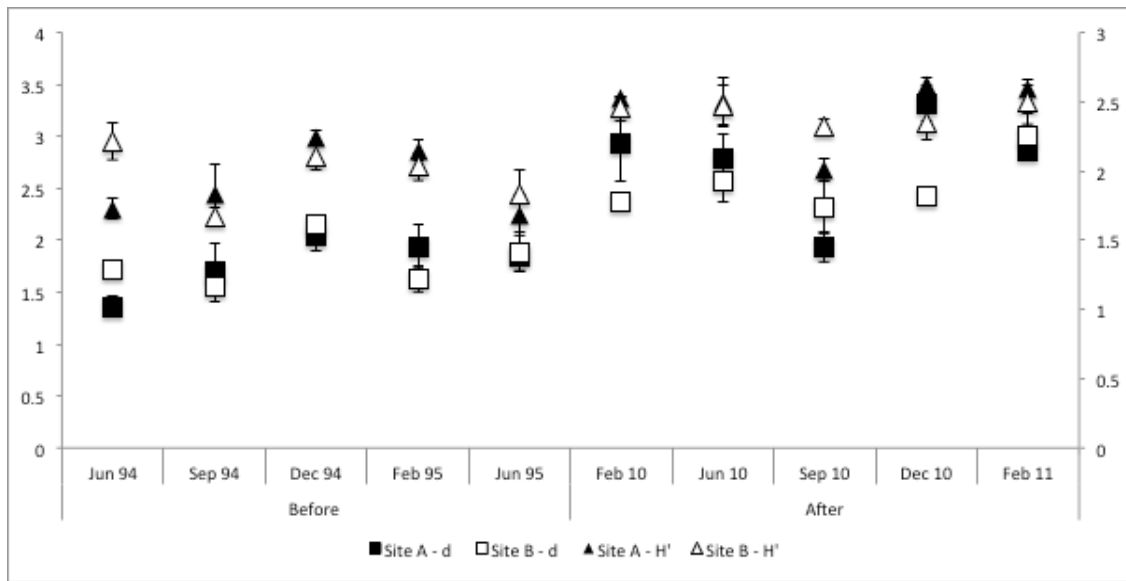


Figure 4. Margalef Index (d) and Shannon-Wiener index (H') average values and standard error (\pm SE) in each sampling occasion: June (1994, 1995 and 2010); September (1994 and 2010); December (1994 and 2010); February (1995, 2010 and 2011) and distribution across the sites (A and B).

Nematodes assemblages- Trophic composition

Before and after the habitat loss, epigrowth feeders (2A) predominated in nematode assemblages (Before- mean percentage \pm SE: site A- $40.0 \pm 4.6\%$; site B- $44.1 \pm 6.0\%$; After- site A- $42.7 \pm 6.2\%$; site B- $30.9 \pm 5.6\%$). Before the collapse, the second most abundant trophic group was the selective deposit feeders (1A) (site A- $26.4 \pm 3.9\%$; site B- $31.3 \pm 7.7\%$), while after the collapse it was the non-selective deposit feeders (2B) (site A- $30.3 \pm 4.2\%$; site B- $34.5 \pm 5.0\%$). The less abundant trophic group was always omnivores/predators (2B) (Before, site A- $4.8 \pm 1.0\%$; site B- $5.6 \pm 1.3\%$; After- site A- $8.7 \pm 1.3\%$; site B- $11.2 \pm 2.5\%$) (Fig. 5). Concerning the temporal variation, before the seagrass disappearing, epigrowth feeders (2A) was the most abundant trophic group in June 1994, September 1994 (site B), December 1994 (sites A, B), February 1995 (site A) and June 1995 (sites A, B). The highest contribution of non-selective deposit feeders

(1B) was found in June 1994 (site A) and selective deposit feeders (1A) peaked in September 1994 (site A) and February 1995 (site B). After the collapse, epigrowth feeders (2A) predominated in February 2010, June 2010 (sites A, B), September 2010, December 2010 and February 2011 (site A). Non-selective deposit feeders (1B) attained the highest percentage in December 2010 and February 2011 (site B) and selective deposit feeders (1A) presented the major contribution in December 2010 (site B). The omnivores/predators (2B) had the less contribution throughout the sampling occasions before and after habitat loss.

PERMANOVA analysis applied to the trophic structure data showed a significant interaction between factor “Time”, “Site” and “Sampling occasions” ($p < 0.05$), nevertheless no significant interaction was detected between factors “Time” x “Site” ($p > 0.05$) (Table 3). The individual pairwise comparisons on interaction factors revealed a high variability among sampling occasions, significant differences were obtained between ($p < 0.05$): *i*) before the collapse, at site A: June 1994 and June 1995; September 1994 and December 1994; September 1994 and June 1994; December 1994 and June 1995; February 1995 and June 1995; At site B: June 1994 and December 1994; June 1994 and June 1995; September 1994 and February 1995; December 1994 and February 1995; February 1995 and June 1995. *ii*) after the collapse, at site A: September 2010 and December 2010; September 2010 and February 2011; At site B, February 2010 and June 2010; February 2010 and February 2011; June 2010 and February 2011; December 2010 and February 2011. The individual pairwise comparisons were performed following the factor design “Site” x “Sampling Occasion”, revealing significant differences before and after the collapse only at site A, between February 1995 and February 2011 ($p < 0.05$).

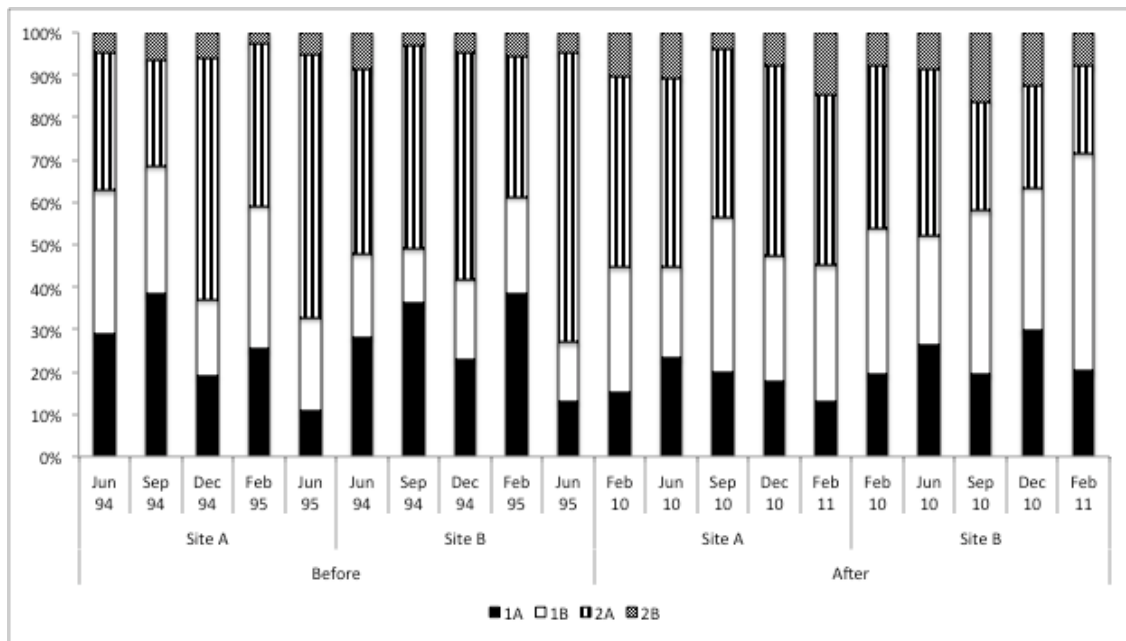


Figure 5. Percentage of contribution of four different trophic groups (1A – selective deposit feeders; 1B – non-selective deposit feeders; 2A – epistrate feeders; 2B – predators), average values in each sampling occasion: June (1994, 1995 and 2010); September (1994 and 2010); December (1994 and 2010); February (1995, 2010 and 2011) and distribution across the sites (A and B).

The index of trophic diversity (ITD) before the collapse ranged from 1.84 ± 0.21 to 3.02 ± 0.17 , and after the collapse the lowest value obtained was 2.78 ± 0.20 and the highest was 3.47 ± 0.26 , indicating the presence of all feeding types (Fig. 6). PERMANOVA analysis of the ITD did not detect significant differences between “Time”, “Site” and “Sampling occasions” ($p > 0.05$) and no significant interactions were shown (Table 3).

The Maturity Index (MI) before the collapse ranged from 2.33 ± 0.03 (site A, June 1994) to 2.66 ± 0.06 (Site B, June 1995). After the collapse it ranged from 2.27 ± 0.07 (site B, September 2010) to 2.59 ± 0.02 (site A, February 2010) and most nematode species showed *c-p* score of 2 (before- 49.7%; After- 65.2%), described by Bongers & Bongers (1998) as ‘general opportunists’, followed by *c-p* score of 3 (before- 49.8%; after- 31.6%) and 4 (before- 0.6%; after- 3.1%) (Fig. 6).

PERMANOVA analysis of MI showed significant differences between sampling occasions (factor “Sampling occasions”, $p < 0.05$). The individual pairwise comparisons of interaction factor “Time” x “Site” x “Sampling occasions” detected a high variability between sampling occasions, namely before the collapse significant differences were obtained between ($p < 0.05$): *i*) at site A- June 1994 and September 1994, June 1994 and December 1994, June 1994 and June 1995, December 1994 and February 1995, February 1995 and June 1995); *ii*) at site B, June 1994 and September 1994, June 1994 and December 1994, June 1994 and June 1995, September 1994 and February 1995, February 1995 and June 1995. After the collapse, at site B no significant differences were obtained between sampling occasions, although at site A there were significant differences between February 2010 and June 2010, February 2010 and September 2010, February 2010 and February 2011 ($p < 0.05$). The individual pairwise comparisons were performed following the factor design “Site” x “Sampling Occasion”, revealing significant differences at site A between February 1995 and February 2010 ($p < 0.05$). At site B significant differences were found between September 1994 and September 2010, December 1994 and December 2010 ($p < 0.05$).

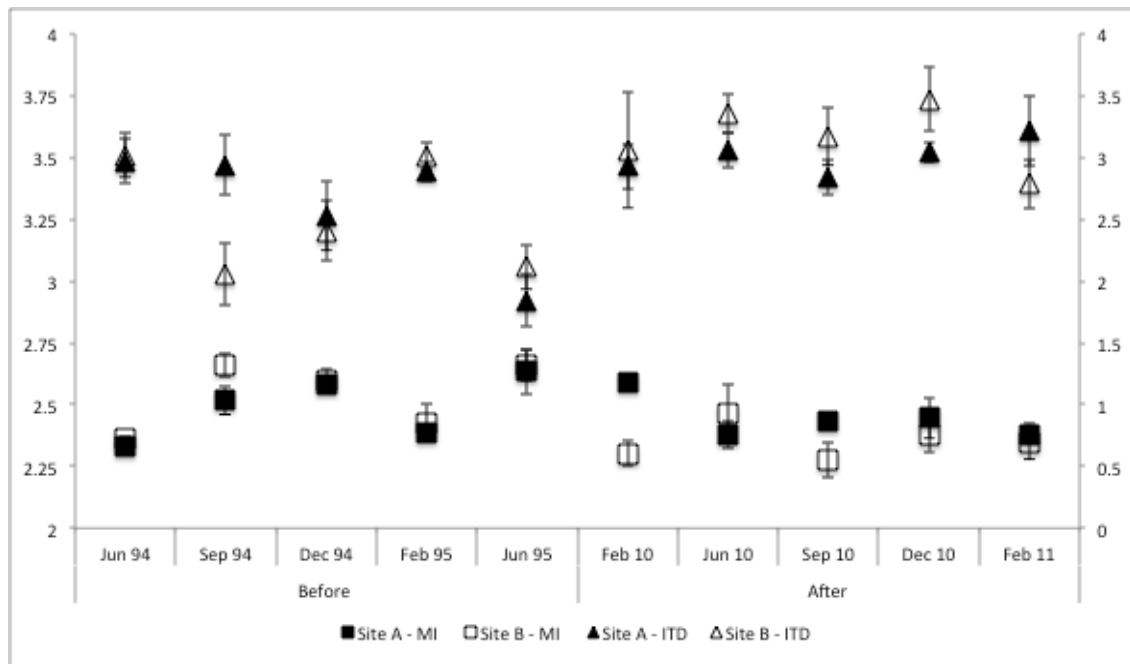


Figure 6. Index of Trophic Diversity (ITD \pm standard error) and Maturity Index (MI \pm standard error), average values in each sampling occasion: June (1994, 1995 and 2010); September (1994 and 2010); December (1994 and 2010); February (1995, 2010 and 2011) and distribution across the sites (A and B).

Nematode assemblage composition

PERMANOVA analysis of the nematodes assemblages density (individuals 10 cm^{-2}) showed significant interactions between factors “Time” x “Site” x “Sampling occasions” ($p < 0.05$), and a significant interaction between factors “Time” x “Site” ($p < 0.05$). Consequently, there were performed individual pairwise comparisons on interaction factors, revealing higher variability between sampling occasions before than during the recovery of the seagrass bed. Significant differences ($p < 0.05$) were detected between: i) before the collapse, at site A: June 1994 and December 1994; February 1995, June 1995 and June 95; September 1994, December 1994, February 1995, September 1994 and December 1994; at site B: June 1994, September 1994, December 1994 and June 1995; February 1995 and September 1994; December 1994, June 1995;

ii) after the collapse, at site A: September 2010 and June 2010, December 2010, February 2011; at site B: February 2010 and February 2011. PERMANOVA analysis of the nematodes assemblages density (individuals 10 cm⁻²) also showed significant differences between sites (factor “Site”, $p < 0.05$). After the collapse, sites A and B showed significant differences whereas before the collapse they did not.

The individual pairwise comparisons, performed following the factor design “Site” x “Sampling Occasion”, showed significant differences between sampling occasions at both times (before and after) ($p < 0.05$): *i*) at site A: June 1994 and June 2010; December 1994 and December 2010; February 1995, February 2010 and February 2011; June 1995 and June 2010; *ii*) at site B- June 1994, June 1995 and June 2010; September 1994 and September 2010; December 1994 and December 2010; February 1995 and February 2010, February 2011.

These results are also supported by PCO ordination plot and clearly reflect a distinct spatial pattern between factors Time (before and after) and temporal differences between sites. Further, it is visible the low variability of nematode communities within sites before the habitat loss, though site A and site B were clearly separated after the habitat loss during the early recovery (Fig. 7).

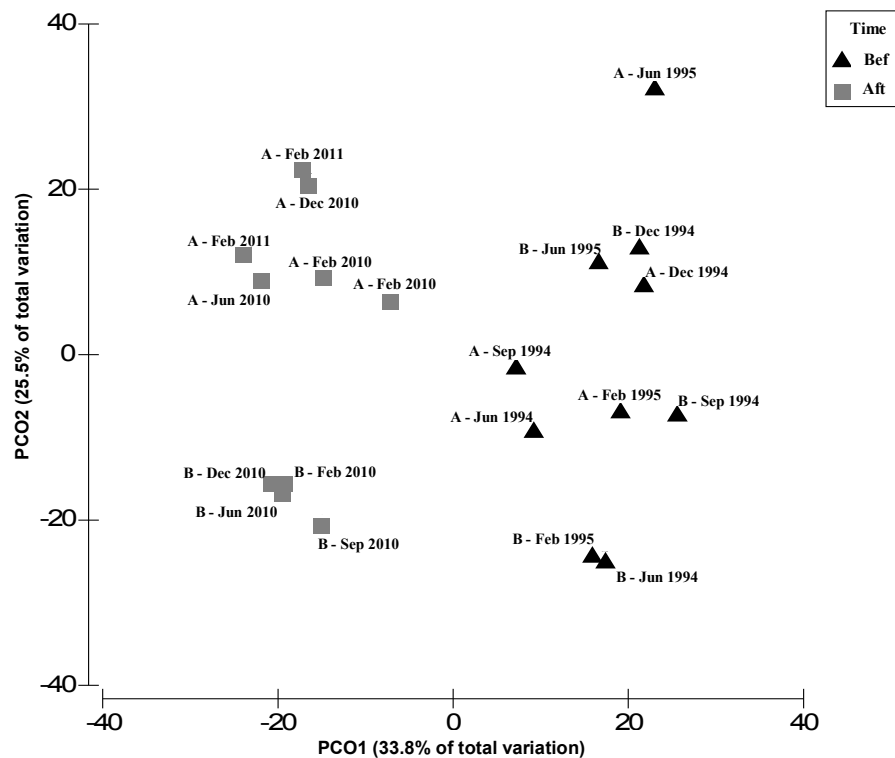


Figure 7. Principal coordinates analysis (PCO) based on nematodes dataset in each “Time” before and after (2 levels, fixed), “Site” A and B (2 levels, random) and “Sampling Occasion” June (1994, 1995 and 2010), September (1994 and 2010), December (1994 and 2010), February (1995, 2010 and 2011) (10 levels, random nested in “Time”). PCO1 = 33.8%, PCO2 = 25.5%.

The SIMPER analysis showed how nematode genera contributed to the similarity values of *a priori* defined groups (Table 4). The genera *Terschellingia*, *Paracomesoma* and *Odontophora* presented the highest contribution to the similarity within before and after the habitat loss (Table 4 – A). From a total of 70 genera, 32 were not present in at least one of the two ecological conditions, before and after the collapse. The genera *Chromadorella*, *Chomadora* and *Paramonohystera* presented higher contribution to the similarity before the collapse, however they were absent after the collapse. On the other hand, the genera *Axonolaimus*, *Promonohystera*, *Anoplostoma* and *Dichromadora*

presented the highest contribution to the similarity during the recovery process and were absent before the collapse (Table 4 – B).

Table 4. Genera determined by (SIMPER) analysis as those most responsible for contributing for the similarities between before and after the collapse of *Z. noltii*, and genera that most contributed for (Dis)similarity between before and after the collapse of *Z. noltii*: A) Distinguishing all genera present in both times (before and After); B) Distinguishing all genera absent before or after.

| A. | | | | B. | | | |
|-------------------------|----------------------|---------------------|----------------------------------|------------------------|----------------------|---------------------|----------------------------------|
| Genera | Before Similarity | After Similarity | Before vs After Dissimilarity | Genera | Before Similarity | After Similarity | Before vs After Dissimilarity |
| | 49% | 55% | 59% | | | | |
| <i>Terschellingia</i> | 21.92 | 16.92 | 7.43 | <i>Axonolaimus</i> | 0 | 6.22 | 3.64 |
| <i>Paracomesoma</i> | 16.92 | 14.56 | 6.52 | <i>Chromadorella</i> | 4.99 | 0 | 2.81 |
| <i>Spirina</i> | 16.05 | 8.83 | 6.11 | <i>Promonhystera</i> | 0 | 2.48 | 1.41 |
| <i>Odonthophora</i> | 15.05 | 11.68 | 4.5 | <i>Chromadora</i> | 2.51 | 0 | 1.33 |
| <i>Ptycholaimellus</i> | 2.8 | 9.19 | 4.13 | <i>Paramonohystera</i> | 1.98 | 0 | 1.1 |
| <i>Sabatieria</i> | 0.16 | 7.57 | 4.06 | <i>Anoplostoma</i> | 0 | 1.96 | 1.09 |
| <i>Linhomoeus</i> | 11.32 | 8.31 | 3.77 | <i>Dichromadora</i> | 0 | 1.92 | 0.99 |
| <i>Metachromadora</i> | 4.06 | 6.2 | 3.59 | <i>Campylaimus</i> | 1.4 | 0 | 0.74 |
| <i>Sphaerolaimus</i> | 1.68 | 6.35 | 3.47 | <i>Megadesmolaimus</i> | 1.2 | 0 | 0.73 |
| <i>Metalinhomeus</i> | 0.52 | 5.86 | 3.33 | <i>Desmodora</i> | 1.58 | 0 | 0.66 |
| <i>Daptonema</i> | 7.62 | 8.73 | 3.12 | <i>Elzalia</i> | 0 | 0.93 | 0.52 |
| <i>Paracyatholaimus</i> | 4 | 5.21 | 2.78 | <i>Paralinhomoeus</i> | 0.81 | 0 | 0.44 |
| <i>Atrochromadora</i> | 0.08 | 4.8 | 2.61 | <i>Southernia</i> | 0.7 | 0 | 0.35 |
| <i>Viscosia</i> | 4.83 | 3.43 | 2.44 | <i>Tricoma</i> | 0 | 0.5 | 0.24 |
| <i>Oncholaimellus</i> | 1.71 | 3.87 | 2.26 | <i>Eurystomina</i> | 0.35 | 0 | 0.22 |
| <i>Chromadorina</i> | 3.45 | 0.11 | 1.89 | <i>Odontanticoma</i> | 0.34 | 0 | 0.22 |
| <i>Eleutherolaimus</i> | 0.12 | 3.41 | 1.84 | <i>Thalassoalaimus</i> | 0 | 0.32 | 0.19 |
| <i>Microlaimus</i> | 1.02 | 2.83 | 1.66 | <i>Euchromadora</i> | 0 | 0.25 | 0.14 |
| <i>Bathylaimus</i> | 2.37 | 1.53 | 1.53 | <i>Crenopharynx</i> | 0 | 0.33 | 0.13 |
| <i>Molgolaimus</i> | 3.14 | 0.13 | 1.47 | <i>Onchium</i> | 0.19 | 0 | 0.12 |
| <i>Halalaimus</i> | 0.24 | 2.34 | 1.4 | <i>Paradesmodora</i> | 0.31 | 0 | 0.12 |
| <i>Prochromadorella</i> | 0.68 | 2.07 | 1.33 | <i>Desmolaimus</i> | 0.17 | 0 | 0.11 |
| <i>Synonchiella</i> | 1.58 | 1.28 | 1.24 | <i>Hypodontolaimus</i> | 0.24 | 0 | 0.09 |
| <i>Camacolaimus</i> | 1.03 | 1.14 | 0.99 | <i>Acanthoncus</i> | 0 | 0.11 | 0.08 |
| <i>Paracanthonchus</i> | 1.41 | 0.5 | 0.89 | <i>Antomicron</i> | 0 | 0.14 | 0.07 |
| <i>Setosabatieria</i> | 0.86 | 1.01 | 0.86 | <i>Chromaspirina</i> | 0 | 0.08 | 0.05 |
| <i>Anticoma</i> | 1.19 | 0.15 | 0.79 | <i>Neochromadora</i> | 0.08 | 0 | 0.05 |
| <i>Oxystomina</i> | 0.63 | 0.86 | 0.76 | <i>Phanodermopsis</i> | 0.07 | 0 | 0.04 |
| <i>Cervonema</i> | 0.07 | 1.35 | 0.69 | <i>Weseria</i> | 0.03 | 0 | 0.03 |
| <i>Comesa</i> | 0.37 | 0.73 | 0.6 | Others | 2.32 | 0 | 1.35 |
| <i>Thalassironus</i> | 0.52 | 0.39 | 0.52 | | | | |
| <i>Leptolaimus</i> | 0.28 | 0.58 | 0.5 | | | | |
| <i>Calyptronema</i> | 0.34 | 0.69 | 0.47 | | | | |
| <i>Nemanema</i> | 0.22 | 0.64 | 0.42 | | | | |
| <i>Diodontolaimus</i> | 0.46 | 0.1 | 0.33 | | | | |
| <i>Cyartonema</i> | 0.19 | 0.41 | 0.32 | | | | |
| <i>Aponema</i> | 0.07 | 0.43 | 0.21 | | | | |
| <i>Aegialoalaimus</i> | 0.14 | 0.29 | 0.19 | | | | |

Separate BIOENV analyses were performed for each sampling occasion in order to analyse the main factors responsible for the distribution of nematode communities before and after the collapse of *Z. noltii*. The combination of six variables: organic matter (MO), biomass of *Z. noltii* (Roots and Leaves), Oxygen percentage (O₂), Chlorophyll *a* (Chl *a*) and silt percentage, accounted for around 90% of the variability within nematode assemblages, using Spearman's rank correlation ($p=0.5$).

Discussion

The estuarine faunal and floral communities are adapted to a high spatial and temporal variability in areas naturally highly stressed, presenting features very similar to those found in areas anthropogenically stressed. These sometimes coincide with those presented under natural stress (Estuarine Quality Paradox) (Dauvin & Ruellet 2009) and because of that the natural and anthropogenic stress effects are often confounded. In essence, estuarine ecological recovery rate and patterns are highly variable. They are greatly influenced by the high ability of the estuarine communities to withstand and recover from natural stressors related with the high variability in transitional waters (Elliott & Whitfield 2011).

The nematode assemblages studied are typical of the intertidal sediments from estuarine euhaline section, organisms which can tolerate adverse and variable environmental conditions. The increase of the ecosystem quality through structural and functional “natural recovery” implies a passive ongoing process which depends on the habitat's potential that may not result in a return to the original state but into a newly created ecosystem regaining quality, resistance and resilience (Elliott et al. 2007). The passive

and natural recovery process of the *Z. noltii* seagrass beds observed in the Mira estuary since 2009, created a rare opportunity to provide critical information and knowledge of the estuarine ecosystem structure and functioning responses to changes. These responses were evaluated during the recovery process towards the original, or newly state, by comparing available former and current data of benthic nematode communities. Indeed, the study of the temporal and spatial distribution patterns of nematodes assemblages during a stable “pre-existing ecological condition” or “state” before the collapse of *Z. noltii* and the early recovery, constituted available baseline data to address the fundamental knowledge to anticipate the trajectories of the recovering ecosystems or even to diagnose.

The environmental characterisation before and after the seagrass disappearance was based on abiotic measurements collected at each sampling event. The characterisation of a system based on chemical parameters only provides information about quality at the time of measurement and does not allow to evaluate the impact of previous events on the ecology of the system (Spellman & Drinan 2001). However, it is useful in providing indications on ecological conditions of the system encompassing the nematode assemblages.

The causes of *Z. noltii* collapse are not yet determined, the absence of visible anthropogenic pressures suggested to relate spatial and temporal patterns of the environmental variables measured mainly with the natural stressors’ characteristics of this estuarine system. The significant decrease of the *Z. noltii* biomass is strongly relevant in the characteristics of the new ecological conditions, such as the decrease of the fine sediments and the increase of the organic matter content of sediments. The presence of the seagrass beds instantly enhances finer sediments and food availability by trapping sediments and nutrients due to the lower physical stress and water

movements (Boström & Bonsdorff 1997). Although in Mira estuary the increase of the grain size sediments could not be related merely with the seagrass loss, the sedimentation dynamic changes observed in last decade show the increase of sandy habitats in Mira estuary (Adão personal communication) that may lead to a seriously drastic impact on seagrass meadows (Cabaço et al. 2008).

The absence of clear temporal patterns of the environmental variables during the stable condition of the seagrass habitat is due to the small range values of temperature, salinity, pH, dissolved oxygen, grain size composition and water nutrients registered. Nevertheless, seagrass biomass registered temporal changes, with higher values in warmer sampling occasions (June, September) and lower in winter months (December, February). In temperate and higher latitude waters, seagrasses have shown to exhibit marked seasonal changes of biomass (Duarte 1989). In Portugal the seasonality of the *Z. noltii* has also been reported in Mira (Ferreira 1994) and Mondego estuaries (Grilo et al. 2012). After the habitat loss, during the early recovery, the biomass of *Z. noltii* registered the lowest values, with absence of the temporal patterns explained by no stable seagrass vegetation patches. Instead, low-biomass patches continually emerged, disappeared and re-appeared at slightly different positions, creating a dynamic mosaic of vegetation patches interspersed with bare sediment patches. The environmental conditions were typical of the intertidal muddy sediments of the estuarine euhaline section, especially the highest salinity values recorded, reflecting a strong dependence on the marine environment (Teixeira et al. 2008).

During the early recovery of the seagrass bed, the nematode density was lower than before the habitat loss, even though the densities were generally high, encompassing a higher number of genera, comparable to those of the estuarine intertidal muddy sediments (Smol et al. 1994; Soetaert et al. 1995; Steyaert et al. 2007). The genera

density, composition and dominance of species obtained before and after habitat loss, in both ecological conditions were in agreement with the nematode assemblages of the temperate and tropical seagrass beds and with euhaline intertidal muddy sediments. These assemblages are commonly cited in the literature as mud-adapted, characterised by higher densities of the genera belonging to the families Linhomoeidae (*Terschellingia*, *Linhomoeus*), Comesomatidae (*Paracomesoma*), Desmodoridae (*Spirinia*) and Axonolaimidae (*Odontophora*) (Wieser 1960; Tietjen 1977; Austen & Warwick 1989; Smol et al. 1994; Soetaert et al. 1995; Ólafsson et al. 2000; Fisher & Sheaves 2003; Rzeznik-Orignac et al. 2003; Steyaert et al. 2003; Johnson et al. 2007; Fonseca et al. 2011). These genera share common characteristics such as tolerance to hypoxic conditions (Jensen 1984; Steyaert et al. 2007) and body morphology that may be advantageous to glide through and over the fine sediments (Warwick 1971), becoming typical in estuarine muddy sediments (Heip et al. 1985). *Terschellingia* and *Paracomesoma* were the two most abundant genera registered before and during the recovery process. They are able to thrive in natural and anthropogenic disturbed habitats (Steyaert et al. 2007; Moreno et al. 2008; Gambi et al. 2009; Armenteros et al. 2009; Alves et al. 2013), including extreme conditions (Moreno et al. 2008; Fonseca et al. 2011).

As expected, during the recovery process, the density of the nematode assemblages decreased. This could be explained due to effects of the strong decrease of the ecosystem engineer *Z. noltii* and to the increase of the proportions of coarser sediments. Many studies have reported that seagrass beds harbour higher abundance, biomass, diversity and productivity of benthic organisms than unvegetated sediments (Edgar et al. 1994; Boström & Bonsdorff 1997; Webster et al. 1998; Hemminga & Duarte 2000; Hirst & Attrill 2008). This has also been shown in several studies concerning

meiobenthic communities, namely nematode assemblages (Alongi 1987; Guerrini et al. 1998; Fisher & Sheaves 2003).

An opposite trend was detected in terms of diversity, species richness and structural diversity, which increased after the habitat loss, during the early recovery process. In this study the lowest biomass of *Z. noltii* was not of the prime importance to explain the nematode diversity during the recovery process. Among the studies analysing meiobenthic communities associated with seagrasses, some also reported slight differences in terms of abundance and diversity and species composition between vegetated and unvegetated sediments (Tietjen 1969; Ndaro & Olafsson 1999; Fonseca et al. 2011), in contrast to macrofaunal assemblages, where unvegetated sediments have reduced the subset of the fauna found in vegetated habitats (Van Houte-Howes et al. 2004). The sedimentary dynamic changes observed in Mira estuary showing a higher proportion of coarse sediments in intertidal habitats, may have contributed to the density decrease and diversity increase because of the wider range of microhabitats available for nematodes in these sediments when compared to muddy ones (Soetaert et al. 2009). Other authors have shown that the diversity of the nematode communities decrease in sediments with a high content of detritus and clay, but the abundance increases (Heip et al. 1985; Coull 1985).

The differences of the nematode species composition and diversity were also explained by the presence and absence of several species, before and after collapse. Thirty-four genera were common to both situations, *Terschellingia*, *Paracomesoma*, *Spirinia*, *Odontophora* and *Ptycholaimellus* presented the greatest contribution for dissimilarity between both nematode assemblages. *Chromadorella*, *Chomadora* and *Paramonohystera* presented the highest contribution for similarity of the assemblages before the collapse, although they were absent after the collapse, while *Axonolaimus*,

Promonhystera, *Anoplostoma* and *Dichromadora* presented the highest contribution for similarity after the collapse but they were absent before the collapse. Studies analysing nematode communities associated with vegetated and unvegetated sediments also detected differences partially explained by the presence and absence of several species (Fonseca et al. 2011).

The epigrowth feeders (2A) were the most abundant trophic group of the nematode assemblages. Although the density decreased and the species diversity increased, the trophic structure remained similar in both ecological conditions, during the stable condition of the seagrass habitat and during the recovery process. The second most abundant group before the collapse was the selective deposit feeders (1A), while after the collapse it was the group of non-selective deposit feeders (1B). The non-selective deposit feeders (1B), epigrowth feeders (2A) and selective deposit feeders (1A) are the most abundant trophic groups in intertidal estuarine muddy sediments and also in seagrass sediments (Escaravage et al. 1989; Soetaert et al. 1994; Soetaert et al. 1995; Chimita & Kikuchi 1996; Danovaro et al. 2002; Rzeznik-Orignac et al. 2003). Frequently, epigrowth feeders (2A) have shown to be the most abundant group in sediments underneath seagrasses (Tietjen 1969; Ndaro & Olafsson 1999; Danovaro & Gambi 2002). Diatoms and other microalgae are important food sources for many representative species of this trophic group (Moens & Vincx 1997). Microphytobenthos (MPB) are an important food source, which often exhibit high production rates in seagrass beds, being available for consuming and being easy digestible (Danovaro et al. 2002; Fisher & Sheaves 2003; Fonseca et al. 2011). The food web structure at both sites (site A; site B) was examined using dual stable isotope signatures, after the habitat loss, by comparing the food sources of macrobenthos and meiobenthos (benthic nematodes and harpacticoid copepods at genus/species level) in the seagrass patches vs in adjacent

unvegetated sediments. The results of this study showed that the organic carbon input of the diet of estuarine macrobenthos and meiobenthic food web in seagrass beds at both sites, derives from various sources, such as seagrass detritus, MPB epiphytic microalgae and suspended particulate organic matter. Moreover, MPB are among the main resources of most nematode taxa, but seagrass-associated resources also contribute to meiobenthos nutrition, with seagrass detritus being available also in deeper sediments and in unvegetated patches close to seagrass beds (Vafeiadou et al. 2013; Vafeiadou et al. 2014). Despite the lower biomass of the *Z. noltii* seagrass, the permanent resources availability proven by this study could explain the similar temporal variation of the nematode trophic composition patterns of the both ecological conditions.

Coupled with taxonomic diversity, functional diversity is important for interpreting distribution patterns of communities (Schratzberger et al. 2008). Specific indicators relying on nematodes information, such as the Maturity Index (MI) and the Trophic Diversity Index (ITD^{-1}), behaved differently. The high values of ITD^{-1} represent high trophic diversity (Moreno et al. 2011) and the low values of MI suggest disturbed habitats, since the opportunistic genera are dominant in adverse conditions (Bongers & Bongers 1998). The high trophic diversity obtained indicates the nematode community responds to good ecological condition of the sediments. On the contrary, the MI results suggest disturbed habitat conditions, a clear dominance of “genera opportunists” (Bongers & Bongers 1998) with *c-p* 2, able to take advantage of disturbed and polluted environments (Gyedu-Ababio & Baird 2006). The sampling sites were located in the euhaline section of the estuary, which are highly naturally stressed because of the high degree of variability in their abiotic characteristics; therefore the structural features of the estuarine communities under this natural stress resemble those of the anthropogenic

stressed areas as defined within the context of the “Estuarine Quality Paradox” (Dauvin & Ruellet 2009).

In temperate regions, intertidal and subtidal meiobenthos are known to vary seasonally, usually with peaks in warmer months (Hicks & Coull 1983; Smol et al. 1994). Moreover, meiobenthic communities seasonal variations are generally more pronounced intertidally than subtidally (Smol et al. 1994; Alves et al. 2013). Nematode assemblages vary seasonally according to the physicochemical regime, the trophic dynamics and the biological factors of the environment. Temporal changes of temperature, salinity, sediment particle size, oxygen, available food resources, trophic interactions, predation, competition and the reproductive burst of the several species have traditionally been implicated in regulating nematodes inhabiting intertidal systems (Alongi 1987; Eskin & Coull 1987; Bouvy & Soyer 1989; Vincx 1989; Ansari & Parulekar 1993; Schizas & Shirley 1996; Ólafsson & Elmegren 1997; Steyaert et al. 1999; Adão 2004). As expected, in intertidal sediments of Mira estuary during both ecological conditions, before and after habitat loss, nematode assemblages density and species composition registered temporal fluctuations, which were strongly pronounced at site A as shown by the temporal pattern based in the MI values. Site A is located very close to the estuary mouth, that is under strong influence of high hydrodynamics and tides regime, these effects are probably modulating nematode assemblages temporal fluctuations and influencing the low densities registered.

Conclusion

Currently, many estuarine and coastal marine ecosystems have an increasing degradation (Halpern et al. 2008), resulting from human activities and natural processes

(Aubry & Elliott 2006). Some ecosystems may never attain the technical definition of being restored and end up irreversibly in an alternative state (Elliott et al. 2006; Borja et al. 2010). The loss of biodiversity that nematode communities are subjected to in stressed environments as organic enrichment, human disturbance and physical stressors, can lead to a reduction in functional biodiversity and might be associated with an exponential decline of ecosystem processes (Mirto & Danovaro 2004; Danovaro et al. 2008; Gambi et al. 2008). In spite of the disturbance caused by the seagrass habitat loss in Mira estuary, the nematode assemblages reveal high resistance and resilience by exhibiting an ability to withstand the natural variability created by the early recovery of *Z. noltii* in the Mira estuary. The nematode community features, such as high abundance and trophic diversity, were typical of estuarine euhaline section, naturally adapted to high stress conditions.

The dataset obtained by comparing the former data of the nematode assemblages as “pre-existing ecological condition” or “state” before the habitat loss and new data of nematode assemblages corresponding to the passive “natural early recovery” habitat allowed to understanding estuarine ecosystem structuring and functioning responses. The essence of ecological functioning was maintained after the habitat loss and it is possible to predict that a “good state” can be achieved if the passive natural recovery process of *Z. noltii* in the Mira estuary continues steadily.

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Chapter III

Biomass and morphometric attributes of nematodes in Mira estuary (Southwest Portugal) before *Zostera noltii* disappearance and during early recovery

Abstract

Biomass and morphometric attributes of nematodes assemblages (body length, width and length/width) in the Mira estuary were analysed before and after a major collapse involving *Zostera noltii* disappearance. It was investigated how biomass and morphometric attributes of nematodes were related to community characteristics and environmental variables. Moreover, biomass and morphometric attributes were investigated for their potential use as complementary tools to classical descriptors such as those based on nematode densities when studying nematodes as biological indicators. Nematode biomass and morphometric attributes proved to be valuable in determining differences in the environmental changes caused by *Z. noltii* collapse. High values of the biomass, length, width and length/width ratio (L/W) were observed after the collapse of *Z. noltii* contrasting with nematode densities values, which showed consistently higher values before the collapse. These results suggest that biomass and allometric attributes of nematodes are indicative of the functional adaptation of nematodes to the new environmental condition in the early recovery process of *Z. noltii* therefore they may be used to provide new and complementary information to assess environmental changes of seagrass ecosystems.

Key words. Nematode morphometry; biomass; *Zostera noltii*; natural recovery; stable condition

Introduction

Free-living nematodes are important members of the meiofauna in marine habitats. They are among the most abundant metazoan organisms, often constituting more than 90% (Schratzberger et al. 2000; Austen & Widdicombe 2006; Alves et al. 2013). They are structurally and functionally diverse (Moens & Vincx 1997), occur in any environment from the most untouched to the most polluted habitats (Coull & Chandler 1992), and respond rapidly to environmental changes (Coull 1999). These variety of characteristics make nematode communities representative of the overall ecosystem status and place them as suitable indicators for detecting changes in environmental conditions over spatial and temporal scales (Coull 1999; Fisher 2003; Norling et al. 2007; Danovaro et al. 2008). The classical nematode community analysis in terms of density, diversity, genera composition and functional diversity is well documented (Castel et al. 1989; Guerrini et al. 1998; Ndaro & Olafsson 1999; Fisher & Sheaves 2003; Fonseca et al. 2011; Alves et al. 2013). However, because these studies rely on taxonomic identification, they are time consuming, expensive and require high levels of expertise.

It is known that nematodes show a wide range of different sizes and body proportions that result from environmental adaptations (Jensen 1984; Vanaverbeke et al. 2004). These morphometric parameters (length, width, length/width ratio and biomass) reflect specific modes of life in terms of feeding strategies, life history, diversity, physiology, energy requirement and biotic and abiotic interactions and may therefore be used to study nematodes ecosystems (Warwick & Price 1979; Soetaert et al. 2002; Vidakovic & Bogut 2004; Moens et al. 2007; Leduc et al. 2010; Quang et al. 2014).

The main causes of the variation in body size seem to be strongly related to biogeochemical conditions of the sediment such as organic content, water content, redox potential, porewater oxygen concentrations and not only to the sediment particle size though frequently attributed exclusively to it (Tita et al. 1999; Vanhove et al. 2004; Fleege et al. 2011). Among other factors that affect nematode morphometry and biomass are food availability, oxygen stress and phytoplankton sedimentation events (Vanaverbeke et al. 2003). It has been predicted that nematode width can increase with increasing particle size in sands (due to the increased size of interstitial spaces) (Wieser 1959; Coull 1988; Tita et al. 1999). However, this can also be due to the fact that sediment pore space can increase food availability creating opportunities for larger organisms (Soetaert et al. 2002; Vanaverbeke et al. 2003).

Length/width ratio (L/W ratio) offers a quantitative measure of their shape (Vanaverbeke et al. 2004). The L/W values may be used to recognize two distinct body shapes as short/stout nematodes with L/W ratios < 6 and long/slender nematodes with L/W ratios > 14 (Ratsimbazafy et al. 1994; Soetaert et al. 2002). Another classification (Schratzberger et al. 2007) considers as stout, nematodes with a low L/W ratio < 18 ; as slender, nematodes with a L/W ratio of 18-72, and as long/thin, nematodes with a high L/W ratio > 72 . Such distinctions can help to understand nematode-sediment relationships.

Assuming that nematode body size and shape can be linked with sediment characteristics and food availability, both biomass and morphometric characteristics become useful descriptors of ecosystems, providing quick and economic responses, with an evident impact on environmental management (Schwinghamer 1983; Soetaert et al. 2002; Chalcraft & Resetarits 2003; Losi et al. 2013).

Many studies have reported that benthic organisms have higher biomass, abundance, diversity and productivity in seagrass beds than in unvegetated sediments (Edgar et al. 1994; Boström & Bonsdorff 1997; Webster et al. 1998; Hemminga & Duarte 2000; Hirst & Attrill 2008). Over the last two decades seagrasses have been particularly vulnerable to natural and anthropogenic pressures such as climate change and its derived effects (Green & Short 2003; Orth et al. 2006; Duarte et al. 2008; Valle et al. 2014). These accelerating pressures have caused seagrass areas disappearance over recent years (Hughes et al. 2009; Waycott et al. 2009). Seagrass beds enhance biodiversity, play an important role in primary production, nutrient cycling, stabilize water flow and promote sedimentation (Orth et al. 2006; Boström et al. 2006) and as a consequence of their disappearance, massive impacts in structural complexity of the habitats have been recorded (Short et al. 2011). Changes on benthic assemblages densities, species composition, trophic composition, as well as on spatial and temporal patterns distributions have been recorded (Boström & Bonsdorff 1997; Boström et al. 2006). Previous studies on an area of seagrass beds of *Zostera noltii* Hornem, performed before and after a major collapse, under vegetated and early recovery circumstances, allowed to establish the general environmental quality condition, using nematode communities descriptors (Materatski et al., in prep., Chapter II; Vafeiadou et al. 2013; Vafeiadou et al. 2014).

The new environmental conditions after *Z. noltii* disappearance, created a dynamic mosaic of *Z. noltii* patches interspersed with bare sediment patches. No stable seagrass vegetation patches emerged, instead, low-biomass patches continually emerged, disappeared and re-appeared at slightly different positions (Materatski et al., in prep., Chapter II). In addition to the effects on the nematode community in terms of density, diversity and trophic composition, the sediment fractions were also clearly

affected by *Z. noltii* mosaic, changing from a dominance of fine sediments to a large proportion of coarser sediments (Materatski et al., in prep., Chapter II).

The aim of the present study was to investigate the nematodes morphometric descriptors, length, width, L/W ratio, and biomass, as complementary information to the classical structural analysis of nematode assemblages, previously detailed (Materatski et al., in prep., Chapter II). To achieve this goal, nematode biomass, length, width and L/W ratio were assessed prior to the *Z. noltii* disappearance – “stable condition” – and in the new environmental conditions, the early recovery process of *Z. noltii*. Additionally, in the two distinct environmental conditions, before and after *Z. noltii* disappearance, the temporal and spatial distribution was analysed at two sampling sites and five sampling occasions. The new environmental conditions were characterised by low *Z. noltii* biomass levels and high grain size sediment. Relationships between nematode biomass, length, width, L/W ratio, and environmental features were investigated.

The following null hypotheses were tested: *i*) there would be no differences on nematodes length, width, L/W, and biomass at different sampling events, before and after *Z. noltii* disappearance; *ii*) there would be no differences on nematodes length, width, L/W, and biomass at different temporal (sampling occasions) and spatial (site A and B) samplings.

Materials and methods

Study area

Sampling was performed in the Mira estuary, south-western coast of Portugal (37°40'N, 8°40'W) (Fig. 1), a small mesotidal system with a semidiurnal tidal regime (amplitude 1-3 m during neap and spring tides, respectively). The estuary has a single

channel, 5–10 m deep and up to 400 m wide, which allows tidal influence to extend 40 km upstream. Due to the low, seasonal and limited freshwater input, the lower section of the estuary has a dominant marine signature characterised, until 2008, by extensive and homogenous *Z. noltii* meadows, with a strong seasonality, with higher biomass in warm months (Cunha et al. 2013). Together with its surrounding area, the Mira estuary is included in a protected area, the Natural Park of ‘Sudoeste Alentejano e Costa Vicentina’. This estuary is considered relatively undisturbed and free from major anthropogenic pressures (Costa et al. 2001). The fluctuations of physico-chemical parameters result mainly from natural pressures as: *i*) its morphology, since the terminal section of the river is rather regular and facilitates the upstream tidal penetration, *ii*) a normally reduced outflow determined by the region’s annual rainfall distribution (concentrated between January and March, with the rest of the year being usually dry) (Paula et al. 2006), and *iii*) the dynamic sedimentation. In 2008, *Z. noltii* meadows disappeared completely. Indications of natural recovery have been observed since 2009 (Cunha et al. 2013; Adão personal communication).

Samples were collected before and after the collapse of *Z. noltii*, during the neap low tide at two sampling sites located in the intertidal sediments; site A, *ca.* 1,5 km from the mouth of the estuary, and site B, 2 km upstream. Sampling collections were carried out at former data on five sampling occasions: June 1994, September 1994, December 1994, February 1995 and June 1995, at each site and sampling occasion two replicates were taken. After the collapse sampling was conducted on five sampling occasions: February 2010, June 2010, September 2010, December 2010 and February 2011, at each site and sampling occasion three replicates were taken.

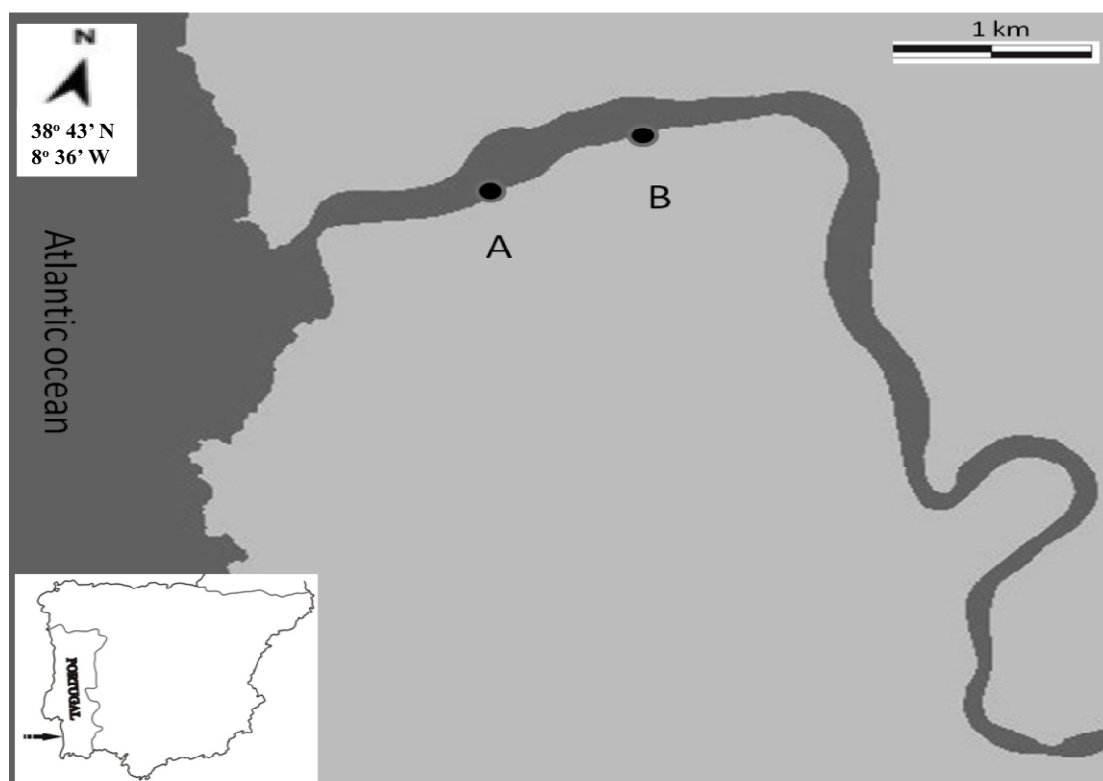


Figure 1. Mira estuary (Portugal): Site locations (black circles). Sites: A, *ca.* 1,5 km from the mouth of the estuary, and B, 2 km upstream.

Environmental data

Salinity, temperature ($^{\circ}\text{C}$), pH, and dissolved oxygen (DO) (mg L^{-1}) of the overlying water just above the sediment were measured *in situ* using a WTW InoLab Multi 720 field probe. Additionally, at each site and on five sampling occasions, water samples were collected in water column for measurement of N and P nutrients ($\mu\text{mol L}^{-1}$) and chlorophyll *a* (mgm^{-3}): nitrate ($\text{NO}_3^{-}\text{-N}$) and nitrite ($\text{NO}_2^{-}\text{-N}$) concentrations were analysed according to standard methods described in Strickland and Parsons (1972) (1972) and ammonium ($\text{NH}_4^{+}\text{-N}$) and phosphate ($\text{PO}_4^{3-}\text{-P}$) concentrations were analysed following the Limnologisk Metodik (1992). Chlorophyll *a* (Chl *a*) determinations were performed according to Parsons et al. (1985). At each site and sampling occasion, sediment samples were taken randomly to determine the organic

matter content (OM) and grain size. Sediment organic matter content was determined based on the difference between the dry weight of each sample after oven-drying at 60°C for 72 h and the weight obtained after combustion at 450°C for 8 h, and was expressed as a percentage of the total weight. The grain size of the sediments was determined with an automatic C.A. Coulter^R LS Particle Size Analyzer. The following size frequency distribution of the sediments was determined: the amount of clay (< 4 µm), the amount of silt (between 4 - 63µm) the amount of sand (> 63µm). The relative content of the different grain size fractions was expressed as a percentage of the total sample weight. *Z. noltii* was collected randomly on each sampling occasion, three replicate samples were taken at each site (A and B) using sediment hand-corers with a surface area of 141 cm⁻² and 30 cm in depth (Marques et al. 1993). On each replicate, the roots were separated from the leaves and then dried in an oven at 60 °C for 48 hours. The leaves and roots biomass was estimated by the organic weight and the ash-free dry weight (AFDW, gm⁻²). AFDW was obtained as the weight loss of the dry material after combustion at 450 °C for 8 hours in a muffle furnace (Heraeus KR 170E).

Nematode assemblages

Nematode samples of the former data (before) were obtained at each sampling site, by forcing hand corers (3,18 cm inner diameter), to a depth of 3 cm, and during the early recovery of the seagrass (after) the replicate sediment samples of the upper 3 cm were also collected using hand corers (4,6 cm inner diameter). All samples were preserved in a 4% buffered formalin solution. Nematodes were extracted from the sediment using a density gradient centrifugation in colloidal silica (Heip et al. 1985). The fixed samples were rinsed on a 1000 µm mesh sieve followed by sieving on a 38 µm mesh.

The fraction retained on the 38 μm sieve was washed and centrifuged three times using the colloidal silica polymer LUDOX HS-40 (specific gravity 1.19). The supernatant of each washing cycle was again collected on a 38 μm sieve. After extraction all nematodes were counted under a stereomicroscope (40 \times magnification). A random set of 120 nematodes was picked from each replicate, transferred through a graded series of glycerol–ethanol solutions, stored in anhydrous glycerol, and mounted on slides (Vincx 1996). Nematodes were identified to genus level using pictorial keys (Platt & Warwick 1988) and the online identification keys/literature available in the Nemys database (Vanaverbeke et al. 2014). Nematode genus level is considered a taxonomic level with good resolution to discriminate disturbance effects (Warwick et al. 1990; Moreno et al. 2008; Schratzberger et al. 2008). Based in total nematode biomass, genera were additionally grouped into the four feeding type groups, designated by Wieser (1953), mainly on the basis of the mouth size and presence or absence of prominent buccal armature: *i*) without a buccal armature that included selective (1A) and non-selective (1B) deposit feeders, and with a buccal armature that included epigrowth feeders (2A) and omnivores/predators (2B). The biomass was estimated using nematode parameters: the nematode length (L) (excluding filiform tail tips) and maximum width (W), which were measured under a Olympus BX-50 compound microscope (1000 \times magnification) with Olympus Cell[^]D software. In order to determine the individual biomass of all specimens, the Andrassy's formula was used to calculate the wet weight (Andrassy 1956). A ratio of 0.25 was assumed to determinate the nematode dry weight (dwt) (Heip et al. 1985). The length/width ratio (L/W) is a quantitative measure of the nematode shapes, calculated with maximum body length/body width ratio (Platt & Warwick 1983; Vanaverbeke et al. 2004; Losi et al. 2013).

Nematode body shape was determined in accordance to Schratzberger et al. (2007) and nematodes were classified on three different classes in terms of morphology as: stout (L/W ratio < 18 μm), slender (L/W ratio of 18-72 μm) and long (L/W ratio > 72 μm).

Data Analysis

Univariate and multivariate analyses to detect temporal and spatial changes in nematode community biomass, length, width and L/W ratio, before the *Z. noltii* disappearance and in early recovery process, were performed using the PRIMER v6 software package (Clarke & Warwick 2001) with the PERMANOVA add-on package (Anderson et al. 2008).

Environmental variables

A Principal Component Analysis (PCA) of the environmental variables measured was performed to find patterns in multidimensional data by reducing the number of dimensions, with minimal loss of information. The PCA ordination was based in the average of the environmental factors in each “Sampling occasions” during “Before” and “After” the habitat loss at each “Sites”. Prior to the calculation of the environmental parameter, resemblance matrix based on Euclidean distances, was log (X+1) transformed followed normalization. Selective transformations were required for the environmental variables water Chlorophyll *a*, nitrate, nitrite, ammonium and phosphate concentrations of the water and sediments, so that the optimal conditions were used for calculating normalized Euclidean distances.

Nematode assemblages

Total nematode biomass (μg individuals 10 cm^{-2}), nematode length, width, length/width ratio, nematode genera composition, and trophic composition based on nematode biomass data were calculated, for each site and sampling occasions before *Z. noltii* disappearance and in the early recovery process. In order to investigate the trophic composition of the assemblages, nematodes biomass genera were assigned to one of four feeding groups according to Wieser (1953), mainly on the basis of the mouth morphology, including presence or absence of prominent buccal armature. A two-way permutational analysis of variance (PERMANOVA) was applied to test the null hypothesis that no significant differences between before *Z. noltii* disappearance and early recovery process, temporal (between sampling occasions) and spatial (between sites) existed in the nematode assemblage descriptors: total biomass, length, width, length/width ratio, genera composition and trophic composition. All PERMANOVA analyses were carried out using the following three-factor design: “Time” before and after (2 levels, fixed); “Site” A and B (2 levels, random) and “Sampling Occasion” June (1994, 1995 and 2010), September (1994 and 2010), December (1994 and 2010), February (1995, 2010 and 2011) (10 levels, random nested in time). Nematode biomass data were fourth-root transformed and the Bray–Curtis similarity was used for calculating the resemblance matrix (Clarke & Green 1988). The null hypothesis was rejected at a significance level <0.05 (if the number of permutations was lower than 150, the Monte Carlo permutation p was used). Whenever significant interactions in effects of the factors were detected, these were examined using posterior pairwise comparisons, using 9999 permutations under a reduced model. The similarity in communities between before and after, sites and sampling occasions were plotted by Principal Coordinates Analysis (PCO) using the Bray-Curtis similarity measure.

The relative contribution of each genus to the average dissimilarities between Time (before and after), was calculated using two way-crossed similarity percentage analysis (SIMPER, cut-off percentage: 90%).

The relationship between environmental variables and the structure of the nematode community was explored by carrying out the BIOENV procedure (Clarke & Ainsworth 1993), using Spearman's correlation.

Results

Environmental variables

Environmental variables measured before and after the major collapse were clearly different. As expected, the biomass of *Z. noltii* was very low and registered strong fluctuations in the early recovery process at site A, ranging from a complete absence in December 2010 to the highest values in June 2010 (7.60 g m^{-2}), and, at site B, ranging from a complete disappearance in June 2010 to highest values (9.50 g m^{-2}) in February 2010. The biomass of *Z. noltii* showed different results before the collapse at site A, where it showed the lowest values in September 1994 (55.04 g m^{-2}) and the highest values in June 1995 (84.03 g m^{-2}). At site B, it showed the lowest values in December 1994 (34.65 g m^{-2}) and the highest values in June 1994 (70.97 g m^{-2}).

Sediment fractions changed drastically after the collapse of *Z. noltii*, at site A and B sediments presented highest proportions of sand ($> 0.063 \text{ mm}$) near to 85%, followed by silt (between $0.004 - 0.063 \text{ mm}$) near to 10% and clay ($< 0.004 \text{ mm}$) near to 5%. Different results were shown before *Z. noltii* disappearance, where sediments registered highest percentage of silt near to 55%, followed by sand near to 35% and clay near to 10%. The PCA ordination of the environmental factors showed that the

two first components (PC1, 36,0% and PC2, 19,0%) accounted for about 60.0% of the variability of data (Fig. 2). The PCA ordination clearly separated the samples collected before and after the collapse, mainly due to the sand sediments, nitrite, nitrate and temperature that present higher values after the collapse.

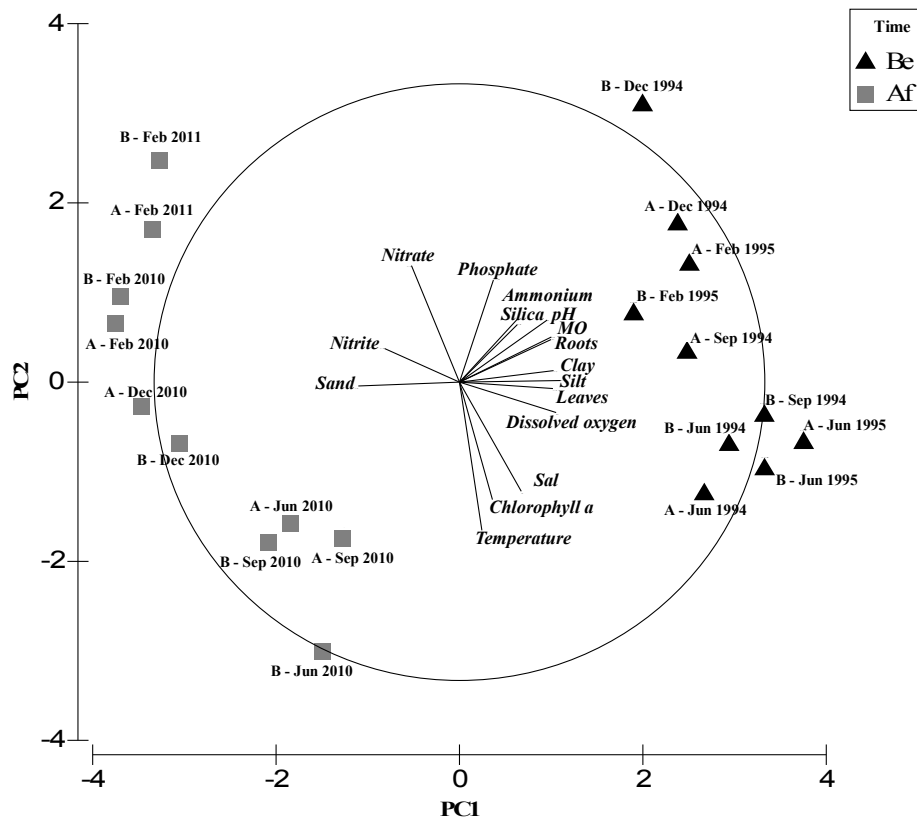


Figure 2. Principal Component Analysis (PCA) plot based on the environmental variables measured in each “Time” before and after (2 levels, fixed), “Site” A and B (2 levels, random) and “Sampling Occasion” June (1994, 1995 and 2010), September (1994 and 2010), December (1994 and 2010), February (1995, 2010 and 2011) (10 levels, random nested in “Time”). PC1 = 52.7%, PC2 = 15.6%.

Nematode community analysis

The nematode community before and after the habitat loss was described in detail in Materatski et al. (in prep., Chapter II). Only the main points based on nematode densities will be reported here. The nematode assemblages density were significantly

higher before *Z. noltii* disappearance than in the early recovery process (PERMANOVA, factor “Time”, $p < 0.05$) as reflected by PCO ordination plot (Fig. 3). Also significant differences were found between sites (PERMANOVA, factor “Site”, $p < 0.05$) and between sampling occasions (PERMANOVA, factor “Sampling occasion”, $p < 0.05$). Before the collapse the mean density (\pm SE) at site A was 1798 ± 180 ind. 10 cm^{-2} ranging from 644 ± 115 ind. 10 cm^{-2} (June 95) to 2628 ± 448 ind. 10 cm^{-2} (February 95). At site B, the mean density (\pm SE) was 3338 ± 517 ind. 10 cm^{-2} and ranged from 1276 ± 279 ind. 10 cm^{-2} (June 95) to 6242 ± 1344 ind. 10 cm^{-2} (February 95). After habitat loss, density of nematodes was lower, at site A the mean density (\pm SE) was 1119 ± 147 ind. 10 cm^{-2} and ranged from 705 ± 86 ind. 10 cm^{-2} (February 11) to 1615 ± 119 ind. 10 cm^{-2} (September 10). At site B, the mean density was 2819 ± 406 ind. 10 cm^{-2} and ranged from 826 ± 169 (February 11) ind. 10 cm^{-2} to 3533 ± 182 ind. 10 cm^{-2} (February 10).

The pre-existing state before habitat loss presented five dominant genera *Terschellingia* (26.8%), *Paracomesoma* (15.4%), *Spirinia* (14.2%), *Odonthophora* (10.8%) and *Linhomoeus* (7.2%) that together comprised nearly 75% of nematode abundances. The nematode community after habitat loss showed that nine dominant genera corresponded to that same 75% of nematode abundances, *Terschellingia* (19.6%), *Paracomesoma* (14.6%), *Odonthophora* (8.5%), *Ptycholaimellus* (6.1%), *Spirinia* (6.0%), *Sabatieria* (5.3%), *Linhomoeus* (5.1%), *Metachromadora* (4.8%) and *Daptonema* (4.7%). The total number of genera found in each time (before and after) was 58 and 50, respectively.

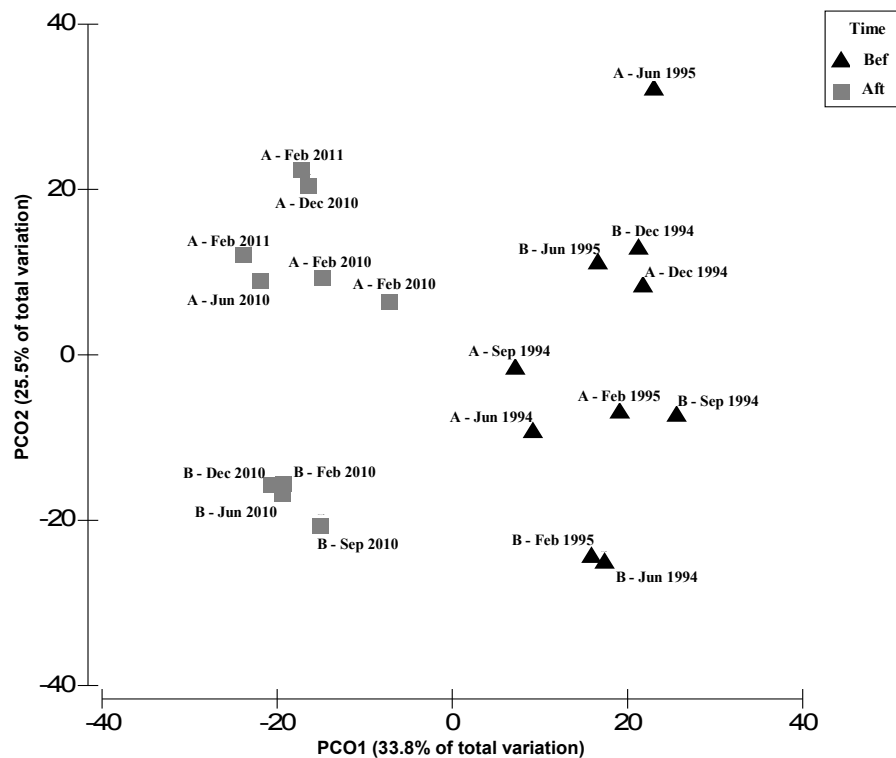


Figure 3. Principal coordinates analysis (PCO) based on nematodes densities dataset in each “Time” before and after (2 levels, fixed), “Site” A and B (2 levels, random) and “Sampling Occasion” June (1994, 1995 and 2010), September (1994 and 2010), December (1994 and 2010), February (1995, 2010 and 2011) (10 levels, random nested in “Time”). PCO1 = 33.8%, PCO2 = 25.5%.

Nematode community analysis based on nematode biomass and diversity

Nematode biomass showed significant differences between before and after the habitat loss (factor “Time”, $p < 0.05$) (Table 2, all PERMANOVA results), with significantly higher values found after the collapse, mean biomass (\pm SE) was $1809 \pm 218 \mu\text{g ind. } 10 \text{ cm}^{-2}$ than pre-existing state, mean biomass (\pm SE) was $1545 \pm 218 \mu\text{g ind. } 10 \text{ cm}^{-2}$ (Fig. 5). Additionally, nematode biomass showed significant differences between sites (factor “Site”, $p < 0.05$). Individual pairwise comparisons on interaction factor (“Time” x “Site” x “Sampling occasions”) before the collapse revealed a low

variability between site A and B, with no significant differences (Pairwise Tests, $p_{A \text{ vs. } B} > 0.221$), at site A the mean biomass (\pm SE) was $1273 \pm 200 \mu\text{g ind. } 10 \text{ cm}^{-2}$ and at site B the mean biomass (\pm SE) was $1816 \pm 373 \mu\text{g ind. } 10 \text{ cm}^{-2}$. In opposition, after the collapse there was a high variability between site A and B with significant differences (Pairwise Tests, $p_{A \text{ vs. } B} < 0.017$), at site A the mean biomass (\pm SE) was $1253 \pm 225 \mu\text{g ind. } 10 \text{ cm}^{-2}$ and at site B the mean biomass (\pm SE) was $2364 \pm 319 \mu\text{g ind. } 10 \text{ cm}^{-2}$, as reflected by PCO ordination plot (Fig. 4).

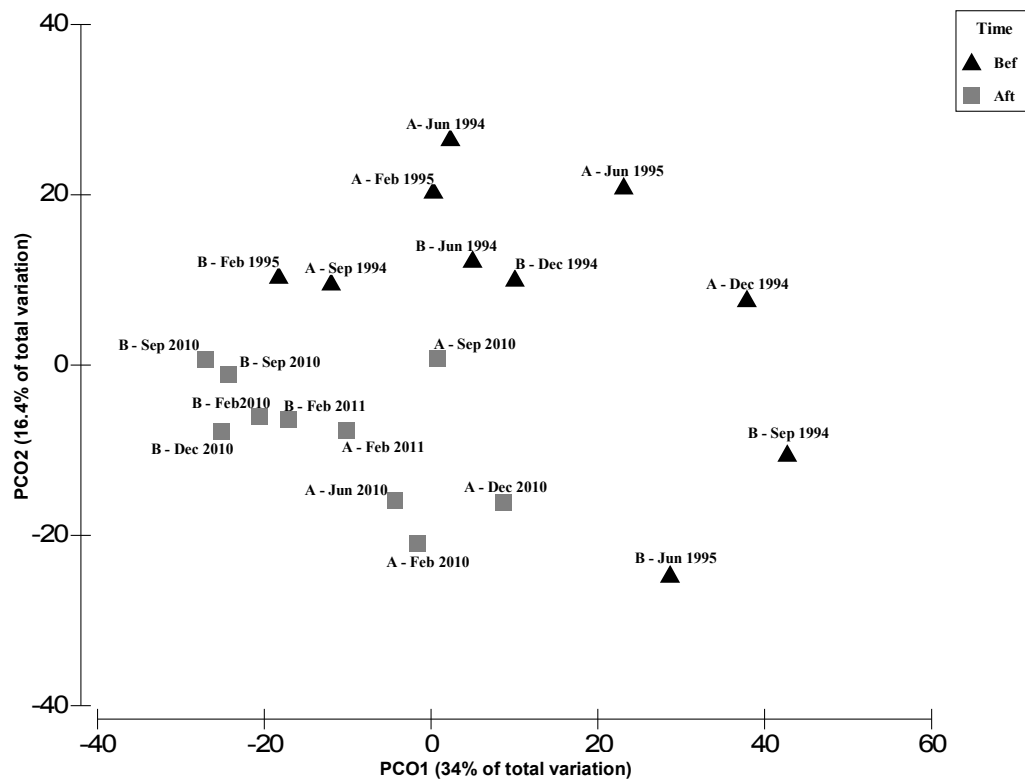


Figure 4. Principal coordinates analysis (PCO) based on nematodes biomass dataset in each “Time” before and after (2 levels, fixed), “Site” A and B (2 levels, random) and “Sampling Occasion” June (1994, 1995 and 2010), September (1994 and 2010), December (1994 and 2010), February (1995, 2010 and 2011) (10 levels, random nested in “Time”). PCO1 = 34%, PCO2 = 16.4%.

Comparing before and after the collapse, the variability of nematodes biomass was

high at both sites (A;B), exhibiting significant differences in both comparisons (Pairwise Tests, $p_{A \text{ (Before) vs. A (After)}} < 0.004$, $p_{B \text{ (Before) vs. B (After)}} < 0.003$). The nematode biomass showed no significant differences between sampling occasions (factor “Sampling occasions”, $p > 0.05$) at both periods (Before and after). Before the collapse of *Z. noltii* at site A, the mean biomass (\pm SE) ranged from $575 \pm 263 \mu\text{g ind. } 10 \text{ cm}^{-2}$ (December 1994) to $1818 \pm 325 \mu\text{g ind. } 10 \text{ cm}^{-2}$ (February 1995). At site B, the mean biomass (\pm SE) ranged from $852 \pm 42 \mu\text{g ind. } 10 \text{ cm}^{-2}$ (September 1994) to $2479 \pm 1999 \mu\text{g ind. } 10 \text{ cm}^{-2}$ (June 1995). After habitat loss biomass of nematodes ranged, at site A, the mean biomass (\pm SE) ranged from $448 \pm 55 \mu\text{g ind. } 10 \text{ cm}^{-2}$ (December 2010) to $1959 \pm 74 \mu\text{g ind. } 10 \text{ cm}^{-2}$ (September 2010). At site B, the mean biomass (\pm SE) ranged from $1059 \pm 110 \mu\text{g ind. } 10 \text{ cm}^{-2}$ (February 2011) to $3337 \pm 1230 \mu\text{g ind. } 10 \text{ cm}^{-2}$ (September 2010) (Fig. 5).

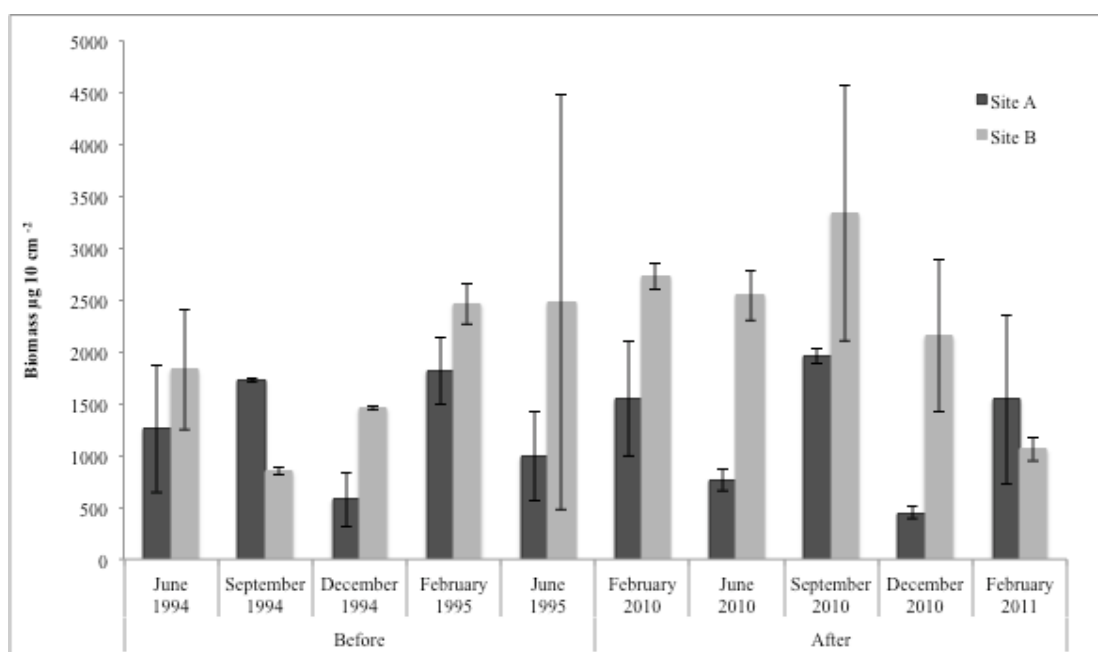


Figure 5. Nematode biomass ($\mu\text{g } 10 \text{ cm}^{-2}$) before and after the collapse, average values and standard error (\pm SE) in each sampling occasion; before (June 1994, September 1994, December 1994, February 1995 and June 1995) and after (February 2010, June 2010, September 2010, December 2010 and February 2011).

September 2010, December 2010, February 2011) in each site (A and B), Before and After the collapse of *Z. noltii*.

The SIMPER analysis based on total nematode biomass showed how nematode genera contributed to similarity values of the *a priori* defined groups (before and after). The genera that most contributed to the similarity before *Z. noltii* disappearance were *Paracomesoma*, *Terschellingia*, *Spirinia*, *Odontophora* and *Linhomus*, while at the early recovery process these were *Paracomesoma*, *Terschellingia*, *Spaerolaimus*, *Linhomus*, *Odontophora*. The genera that contributed most to the dissimilarities between before and after *Z. noltii* disappearance were *Spaerolaimus*, *Sabatieria*, *Axonolaimus*, *Spirinia*, *Paracomesoma* and *Metachromadora* (Table 1).

Table 1. Genera determined by (SIMPER) analysis as those most responsible for contributing for the Similarities within before and within after the collapse of *Z. noltii*, and genera that most contributed for (Dis)similarity between before and after the collapse of *Z. noltii*. The table only lists all nematode genera which contribute with at least 1.5%.

| | Before | After | Before vs After |
|-------------------------|------------|-------|-----------------|
| Genera | Similarity | | Dissimilarity |
| | 49% | 55% | 54% |
| <i>Sphaerolaimus</i> | 0.96 | 3.11 | 6.15 |
| <i>Sabatieria</i> | 0 | 2.26 | 4.98 |
| <i>Axonolaimus</i> | 0 | 2.15 | 4.89 |
| <i>Spirinia</i> | 3.31 | 2.72 | 4.21 |
| <i>Paracomesoma</i> | 3.83 | 4.29 | 4.06 |
| <i>Metachromadora</i> | 0.97 | 1.62 | 3.55 |
| <i>Oncholaimellus</i> | 0.49 | 1.36 | 3.11 |
| <i>Metalinhomoeus</i> | 0.33 | 1.45 | 3.05 |
| <i>Viscosia</i> | 1.19 | 1.32 | 2.92 |
| <i>Ptycholaimellus</i> | 0.92 | 1.93 | 2.78 |
| <i>Paracyatholaimus</i> | 1.5 | 1.57 | 2.78 |
| <i>Linhomoeus</i> | 2.93 | 2.83 | 2.7 |
| <i>Terschellingia</i> | 3.19 | 3.21 | 2.61 |
| <i>Atrochromadora</i> | 0.07 | 1.19 | 2.6 |
| <i>Bathylaimus</i> | 0.78 | 0.82 | 2.44 |
| <i>Eleutherolaimus</i> | 0 | 1.06 | 2.31 |
| <i>Daptonema</i> | 1.84 | 2.52 | 2.29 |
| <i>Synonchiella</i> | 0.68 | 0.62 | 2.16 |
| <i>Chromadorella</i> | 0.96 | 0 | 2.15 |
| <i>Promonhystera</i> | 0 | 0.91 | 2.02 |
| <i>Molgolaimus</i> | 0.9 | 0.03 | 1.91 |
| <i>Odontophora</i> | 2.75 | 2.49 | 1.77 |
| SpA | 0.76 | 0 | 1.71 |
| <i>Camacolaimus</i> | 0.53 | 0.41 | 1.7 |
| <i>Halalaimus</i> | 0.13 | 0.68 | 1.59 |
| <i>Chromadorina</i> | 0.69 | 0.03 | 1.53 |
| <i>Oxystomina</i> | 0.35 | 0.44 | 1.5 |
| Other Genera | 5.51 | 6.17 | 24.35 |

Before and after the collapse nematode assemblages showed clear differences among the dominant genera, although all the dominant genera were present in both ecological conditions. Before the collapse nearly 80% of the nematode community biomass was

represented by only six genera, *Paracomesoma* (28.0%), *Spirinia* (23.8%), *Terschellingia* (10.5%), *Linhomoeus* (8.0%), *Odonthophora* (5.0%) and *Sphaerolaimus* (4.2%). After the collapse eight genera corresponded to 80% of nematode community biomass, *Paracomesoma* (32.1%), *Sphaerolaimus* (12.3%), *Terschellingia* (9.2%), *Spirinia* (9.0%), *Linhomoeus* (6.7%), *Sabatieria* (5.3%), *Metachromadora* (3.6%) and *Odonthophora* (3.5%).

The distribution of the feeding groups based on nematodes biomass data revealed different predominances in nematode assemblages, before and after the habitat loss. The composition of the feeding groups during the ecological stable condition, registered at site A and B, the epistrate feeders, 2A, with the highest biomass in nematode assemblages, mean percentage (\pm SE), (site A- $22.2 \pm 4.9\%$; site B- $55.1 \pm 19.3\%$), followed by non-selective deposit feeders, 1B (site A- $50.4 \pm 13.6\%$; site B- $24.4 \pm 9.5\%$), selective deposit feeders, 1A (site A- $20.0 \pm 6.6\%$; site B- $9.8 \pm 2.5\%$) and omnivores/predators, 2B (site A- $7.3 \pm 4.7\%$; site B- $10.6 \pm 4.4\%$). After the collapse, during the early recovery, the feeding groups based on biomass values presented predominances, at site A and B in nematode assemblages of non-selective deposit feeders 1B, that registered the highest biomass, the mean percentage (\pm SE), (site A- $43.8 \pm 14.4\%$; site B- $47.2 \pm 7.3\%$), followed by epistrate feeders, 2A (site A- $31.8 \pm 6.6\%$; site B- $19.2 \pm 3.4\%$), omnivores/predators, 2B (site A- $14.6 \pm 3.3\%$; site B- $21.8 \pm 5.7\%$) and selective deposit feeders, 1A (site A- $9.7 \pm 2.5\%$; site B- $11.6 \pm 2.3\%$) (Fig. 6). PERMANOVA analysis of the feeding groups based in nematode biomass did not detect any significant differences between before and after habitat loss (factor “Time” $p > 0.05$), sites (factor “Site” $p > 0.05$) or sampling occasions (factor “Sampling occasion” $p > 0.05$). Individual pairwise comparisons on interaction factor “Time” x “Site” x “Sampling occasions”) before the seagrass

disappearance revealed a low variability of the biomass values, between sites A and B no significant differences was obtained (Pairwise Tests, $p_{A \text{ vs. } B} > 0.162$). After the collapse, between site A and B, it was also registered a low variability and there were not detected significant differences (Pairwise Tests, $p_{A \text{ vs. } B} > 0.056$). Individual pairwise comparisons between before and after habitat loss, at site A, revealed low variability and no significant differences (Pairwise Tests, $p_{A \text{ (Before) vs. } A \text{ (After)}} > 0.058$). Individual pairwise comparisons between before and after, at site B, revealed significant differences (Pairwise Tests, $p_{B \text{ (Before) vs. } B \text{ (After)}} < 0.025$).

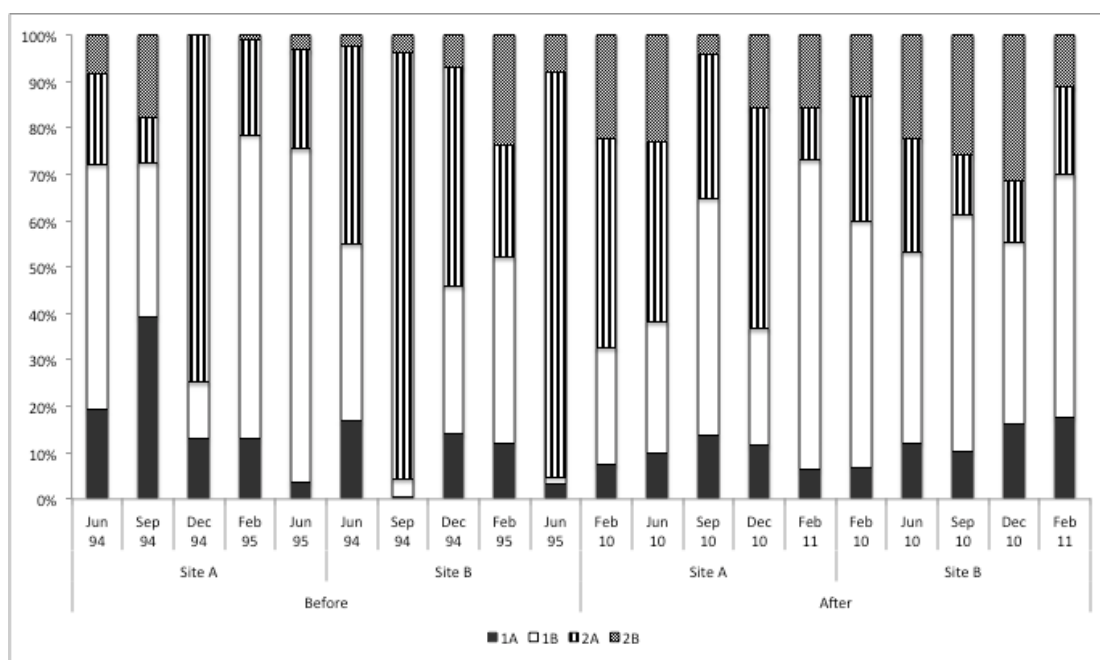


Figure 6. Percentage of contribution based on nematode biomass data of the four different trophic groups (1A – selective deposit feeders; 1B – non-selective deposit feeders; 2A – epistrate feeders; 2B – predators), in each Time (before and after), Site (A and B) and Sampling Occasion (June 1994, 1995 and 2010; September 1994 and 2010; December 1994 and 2010; February 1995, 2010 and 2011).

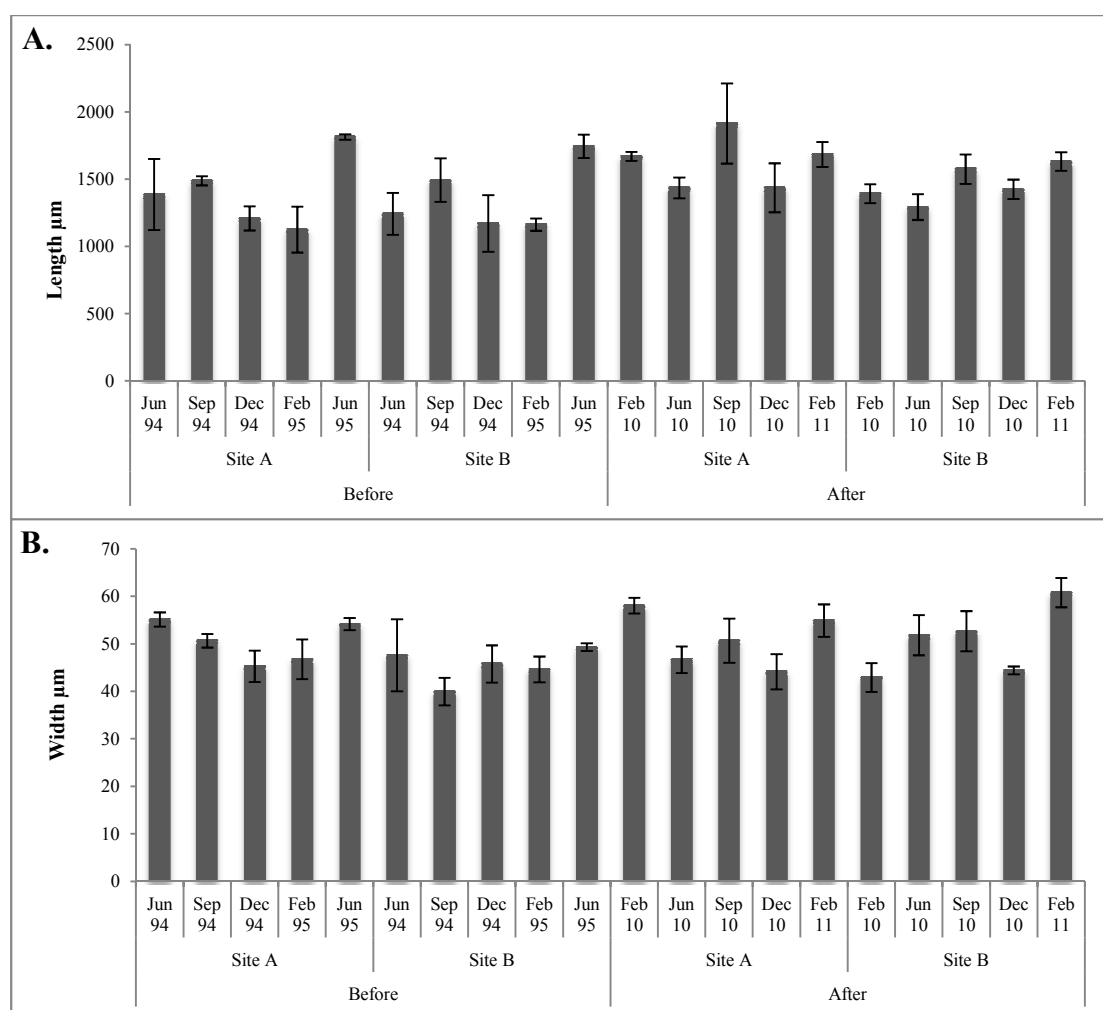
Nematode morphometric attributes

The relative frequency distribution of the nematode dimensions (length/width) showed that slender nematodes (L/W ratio of 18-72) were more prevalent before and after the habitat loss, although with slight differences 90.5% and 85.2%, respectively. Slight differences were also found in the other two morphological groups, with higher values of long nematodes (L/W ratio > 72) after the collapse (9.3%) when compared to before the collapse (4.1%). At both times (before and after) stout nematodes presented the lowest contribution (5.4% and 5.5%, respectively). The mean L/W ratio values (\pm SE) were 29.9 ± 0.9 μ m before and 33.4 ± 0.7 μ m after the collapse. Before the collapse, the largest nematodes belonged to the genus *Linhomoeus* with a length of 4976 μ m and a width of 51 μ m (L/W ratio 97) and the shortest to the genus *Daptonema* with a length of 409 μ m and a width of 51 μ m (L/W ratio 8). After the collapse, during the early recovery, the largest nematodes belonged to the genus *Cyartonema* with a length of 10392 μ m and a width of 94 μ m (L/W ratio 110) and the shortest to the genus *Spirinia* with a length of 113 μ m and a width of 58 μ m (L/W ratio 1.9). The most abundant genera on both sampling periods increased the L/W ratio, except of the *Linhomoeus* genus, *Paracomesoma* (before: 25.9 ± 1.6 ; after: 29.8 ± 0.8), *Spirinia* (before: 30.5 ± 1.2 ; after: 31.5 ± 1.1), *Sphaerolaimus* (before: 12.2 ± 0.7 ; after: 20.4 ± 2.5), *Terschellingia* (before: 25.5 ± 1.0 ; after: 29.0 ± 1.0) and *Linhomoeus* (before: 51.6 ± 2.3 ; after: 50.4 ± 2.5). The allometric parameter L/W ratio of the nematodes was significant higher during the early recovery (factor “Time”, $p < 0.05$). Before the habitat loss, at site A, the mean L/W ratio values (\pm SE) ranged from 24.3 ± 0.5 in February 1995 to 34.1 ± 0.2 in June 1995 and at site B ranged from 26.3 ± 2.5 in December 1994 to 36.1 ± 0.5 in September 1994. During the early recovery, at site A, the L/W ratio mean values (\pm SE) ranged from 32.2 ± 0.7 in February 2010 to 39.8 ± 2.2 in September 2010 and at site B ranged from 27.4 ± 0.6 in June 2010 to

36.3 \pm 1.8 in December 2010 (Fig. 7). The L/W ratio distribution did not show significant differences between sites (factor “Site”, $p > 0.05$) nor among sampling occasions (factor “Sampling occasions”, $p > 0.05$). Individual pairwise comparisons on interaction factors (“Time”, “Site” and “Sampling occasion”) did not detect any significant differences.

The body width distribution of the nematodes assemblages showed significant higher values during the early recovery (factor “Time”, $p < 0.05$). Before the seagrass disappearance, at site A, the width spectra mean values (\pm SE) ranged from 45.2 \pm 3.3 μ m in December 1994 to 55.1 \pm 1.5 μ m in June 1994 and at site B ranged from 39.9 \pm 2.9 μ m in September 1994 to 49.3 \pm 0.8 μ m in June 1994. During the early recovery, width spectra mean values (\pm SE) ranged from 44.1 \pm 3.7 μ m in December 2010 to 58.9 \pm 1.6 μ m in February 2010 and at site B, ranged from 42.9 \pm 3.0 μ m in February 2010 to 60.8 \pm 3.1 μ m in February 2011 (Fig. 7). The width distribution did not show any significant differences between sites (factor “Site”, $p > 0.05$) or among sampling occasions (factor “Sampling occasions”, $p > 0.05$). Individual pairwise comparisons on interaction factors (“Time”, “Site” and “Sampling occasion”) showed significant differences at site A during the early recovery, between February 2010 and June 2010, between February 2010 and September 2010 and between September 2010 and February 2011 (all Pairwise Tests, $p < 0.05$). The length spectra of the nematode genera was significant higher during the early recovery (factor “Time”, $p < 0.05$). Before habitat loss at site A, the length mean values (\pm SE) of the nematodes ranged from 1224.9 \pm 170.8 μ m in February 1995 to 1813.4 \pm 20.0 μ m and at site B ranged from 1162.3 \pm 46.3 μ m in February 1995 to 1744.4 \pm 86.7 μ m in June 1995. During the early recovery, at site A the width mean values of the nematodes (\pm SE) ranged from 1435.0 \pm 78.0 μ m in June 2010 to 1914.3 \pm 298.3 μ m in September 2010 and at

site B, ranged from 1292.4 ± 95.3 in June 2010 to 1613.3 ± 68.6 in February 2011 (Fig. 7). The length distribution of the nematodes assemblages did not show any significant differences between sites (factor “Site”, $p > 0.05$) or among sampling occasions (factor “Sampling occasions”, $p > 0.05$). Individual pairwise comparisons on interaction factors (“Time”, “Site” and “Sampling occasion”) detected significant differences at site B before the habitat loss between June 1994 and September 1994 (Pairwise Tests, $p < 0.05$). During the early recovery at site A the individual pairwise comparisons showed significant differences, between February 2010 and September 2010, between September 2010 and February 2011 (all Pairwise Tests, $p < 0.05$).



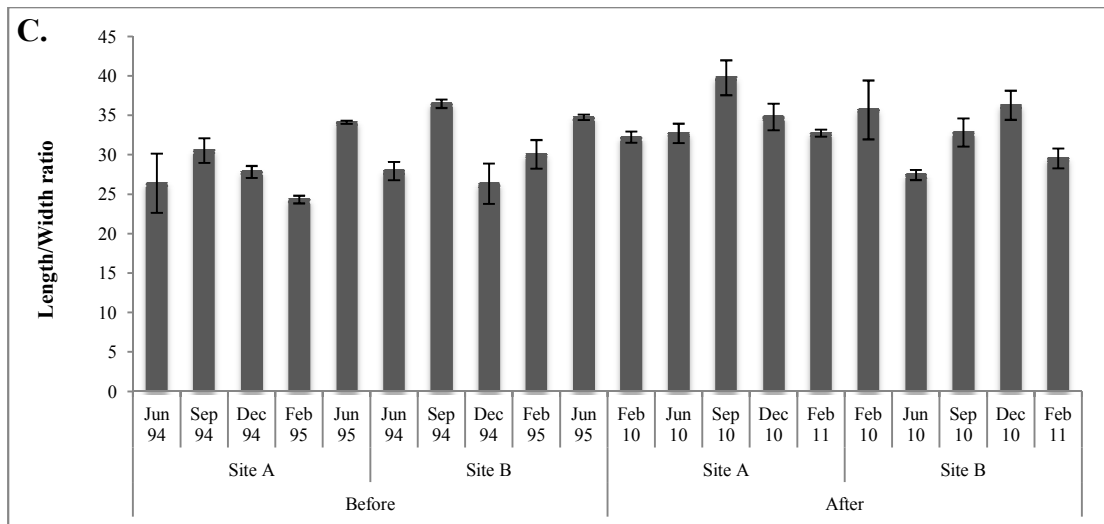


Figure 7. Nematode morphometric parameters: A. Length; B. Width; C. Length/Width ratio, in each Time (before and after), Site (A and B) and Sampling Occasion (June 1994, 1995 and 2010; September 1994 and 2010; December 1994 and 2010; February 1995, 2010 and 2011).

Table 2. Details of the three-factor PERMANOVA test in each “Time” before and after (2 levels, fixed), “Site” A and B (2 levels, random) and “Sampling Occasion” June (1994, 1995 and 2010), September (1994 and 2010), December (1994 and 2010), February (1995, 2010 and 2011) (10 levels, random nested in “Time”), for all variables analysed. Bold values stand for the significant differences ($p < 0.05$).

| | Source of variation | Degrees of freedom | Sum of squares | Mean squares | Pseudo-F | perms | P(perm) |
|---------------------|---------------------------------|--------------------|----------------|--------------|----------|-------|---------------|
| Nematode biomass | Time | 1 | 12746 | 12746 | 3.3392 | 9943 | 0.0187 |
| | Site | 1 | 3058.5 | 3058.5 | 2.5219 | 9928 | 0.0365 |
| | Sampling Occasion (Time) | 8 | 11275 | 1409.3 | 1.1389 | 9855 | 0.2885 |
| | Time x Site | 1 | 2820 | 2820 | 2.3251 | 9933 | 0.0391 |
| | Site x Sampling Occasion (Time) | 8 | 9899.3 | 1237.4 | 1.9877 | 9810 | 0.0001 |
| | Residual | 30 | 18676 | 622.54 | | | |
| | Total | 49 | 58747 | | | | |
| Trophic composition | Time | 1 | 555.4 | 555.4 | 0.86311 | 9959 | 0.5848 |
| | Site | 1 | 790.3 | 790.3 | 2.8501 | 9934 | 0.0509 |
| | Sampling Occasion (Time) | 8 | 1274.1 | 159.27 | 0.5595 | 9942 | 0.8879 |
| | Time x Site | 1 | 807.2 | 807.2 | 2.911 | 9942 | 0.0678 |
| | Site x Sampling Occasion (Time) | 8 | 2277.3 | 284.66 | 2.8354 | 9914 | 0.0002 |
| | Residual | 30 | 3011.8 | 100.39 | | | |
| | Total | 49 | 8698.3 | | | | |
| Width | Time | 1 | 16024 | 16024 | 4.843 | 9930 | 0.0038 |
| | Site | 1 | 2530 | 2530 | 1.6504 | 9918 | 0.1313 |
| | Sampling Occasion (Time) | 8 | 11902 | 1487.7 | 0.95107 | 9840 | 0.5969 |
| | Time x Site | 1 | 2190.5 | 2190.5 | 1.4289 | 9931 | 0.2156 |
| | Site x Sampling Occasion (Time) | 8 | 12514 | 1564.3 | 1.9984 | 9836 | 0.0001 |
| | Residual | 30 | 23483 | 782.75 | | | |
| | Total | 49 | 68854 | | | | |
| Length | Time | 1 | 15548 | 15548 | 5.9626 | 9942 | 0.0023 |
| | Site | 1 | 2019.6 | 2019.6 | 1.8891 | 9941 | 0.1034 |
| | Sampling Occasion (Time) | 8 | 9319.1 | 1164.9 | 1.0669 | 9877 | 0.4029 |
| | Time x Site | 1 | 1665.1 | 1665.1 | 1.5576 | 9916 | 0.1729 |
| | Site x Sampling Occasion (Time) | 8 | 8734.4 | 1091.8 | 2.0873 | 9854 | 0.0001 |
| | Residual | 30 | 15692 | 523.07 | | | |
| | Total | 49 | 53071 | | | | |
| L/W ratio | Time | 1 | 15800 | 15800 | 6.0197 | 9936 | 0.0021 |
| | Site | 1 | 2054.9 | 2054.9 | 1.9699 | 9930 | 0.0911 |
| | Sampling Occasion (Time) | 8 | 9329.3 | 1166.2 | 1.0947 | 9907 | 0.3599 |
| | Time x Site | 1 | 1675 | 1675 | 1.6058 | 9930 | 0.1679 |
| | Site x Sampling Occasion (Time) | 8 | 8522.2 | 1065.3 | 2.082 | 9863 | 0.0001 |
| | Residual | 30 | 15350 | 511.67 | | | |
| | Total | 49 | 52816 | | | | |

Separate BIOENV analyses were performed for each sampling occasion in order to determine the main factors responsible for the distribution of nematode communities before and after the collapse of *Z. noltii*. The combination of six variables: Sand,

biomass of *Z. noltii* (roots), biomass of *Z. noltii* (leaves), phosphate, silt percentage and organic matter (MO) accounted for around 90% of the variability within nematode assemblages, using Spearman's rank correlation ($p=0.8$).

Discussion

The classical descriptors of nematode communities such as density, species composition and trophic groups provide important information in terms of detecting natural changes and anthropogenic effects on communities (Castel et al. 1989; Guerrini et al. 1998; Ndaró & Olafsson 1999; Fisher & Sheaves 2003; Fonseca et al. 2011; Alves et al. 2013). However, since several ecological features are influenced by the organisms' size (e.g., metabolic rates, tolerance to chemical stress, mobility, vulnerability to predation), body dimensions and shape may be used as complementary tools to describe important functional attributes of free-living nematode species and genera (Schratzberger et al. 2007; Fleege et al. 2011; Losi et al. 2013; Alves et al. 2014). Morphometric attributes have presented a large information content comparable to community composition based on density with a revived interest in the size distribution of invertebrate communities (Soetaert et al. 2002; Tita et al. 2002; Fleege et al. 2011; Losi et al. 2013; Alves et al. 2014). This research compares the temporal and spatial distribution patterns of the nematode communities biomass based on former data corresponding to stable ecological status, and during the natural early recovery of *Z. noltii* seagrass beds in Mira estuary, "before" and "after" habitat loss. The dataset obtained allowed to analyse the information content of the biomass and morphometric attributes of nematode assemblages and to understand how they reflect the ecological conditions and how

they can be related with the information available on community composition and density.

The comparison of nematode biomass and morphometric characteristics between before *Z. noltii* disappearance and during the early recovery process showed that this extreme event had an effect in nematode species. The *Z. noltii* biomass and sediment fractions were clearly different throughout the studied period (before and after *Z. noltii* disappearance). These results suggest that the seagrass recovery process, characterised by dense patches alternated with very sparse or even non vegetation, significantly modified the ecological conditions of the sediment. This appears as no surprise as seagrass beds are described as ecosystems engineers due to their potential to alter sediment granulometry, stabilize sediments, trap detritus, influence sediment chemistry and alter the microbial and microphytic communities within sediments (Orth et al. 2006; Boström et al. 2006; Wright & Jones 2006; Moreno et al. 2008; Fonseca et al. 2011; Valle et al. 2014). In the last decade, important changes on the sediment dynamics were registered in several Portuguese estuaries, including Mira estuary, which led to drastic impacts on seagrass meadows (Cabaço et al. 2008; Cunha et al. 2013; Adão personal communication).

The BIOENV results showed that the distribution pattern of nematodes in the early recovery process was mainly structured by distinct environmental factors such as sand, biomass of *Z. noltii* (Roots and Leaves), phosphate, silt percentage and organic matter. The study of Materatski et al. (in prep., Chapter II), demonstrated that changes caused by the presence of seagrass on sediment had a pronounced effect and were probably modulating nematode assemblages in terms of density, genera composition and diversity. Nematode density was consistently higher before the collapse of *Z. noltii* however, diversity was higher after the collapse and no significant differences

were found between before and after the collapse in relation to the trophic composition (Materatski et al., in prep. Chapter II). The nematode assemblages density registered an opposite trend to the total nematode biomass that showed highest biomass values during early recovery process. During the ecological stable condition of the seagrass beds, before the collapse, the mean biomass was lower than after the collapse. These values were higher than those observed by Tita et al. (2002) ranging from 96 ± 14 to $248 \pm 86 \mu\text{g } 10 \text{ cm}^{-2}$ but are in agreement with Smol et al. (1994) who showed nematode total biomass values ranged from 49 to 7044 $\mu\text{g } 10 \text{ cm}^{-2}$, both studies performed in intertidal estuaries. To our knowledge, until now there are no studies on nematode biomass and morphometric attributes in intertidal seagrass beds, for this reason studies on intertidal muddy or sand sediments were used as reference for comparative studies.

The increased nematode assemblages biomass observed during the habitat recovery and the decreased densities, could be related to the new ecological state, the early recovery, in which the structure of the nematode assemblages changed in terms of diversity, in a clear favour of larger nematodes. Heip et al. (1985) and Soetaert et al. (2009) mentioned that larger nematodes were associated with coarser grain size. This is confirmed in this study that shows an increase of biomass values, due to the contribution of larger nematodes belonging to genera *Sphaerolaimus*, *Sabatieria* and *Metachromadora*, during the recovery process, characterised by an increase of the grain size. Despite the high densities of the *Terschellingia* populations, these have a low contribution to total biomass due to their small size. *Terschellingia* genus members are described as colonizers with a high reproductive rate, small size and a strong tolerance to extreme conditions (Moreno et al. 2011). The genera *Paracomesoma*, *Terschellingia* and *Spirinia* are typical in intertidal seagrass muddy,

since they tolerate anoxic conditions and organically rich and muddy sediments (Vincx 1989; Vincx 1990; Heip et al. 1990; Vanreusel 1990; Vanreusel 1991; Steyaert et al. 1999; Boyd et al. 2000; Adão 2004; Schratzberger et al. 2006). Although the other abundant genera, *Linhomoeus*, *Sabatieria*, *Metachromadora*, and *Odonthophora*, have been described in intertidal seagrass muddy, their high densities after the collapse may be related with the increase of bare sediment areas (Tita et al. 1999; Adão 2004). The rising of *Sphaerolaimus*, *Sabatieria*, *Axonolaimus*, and *Metachromadora* in the early recovery is supported by the results of the SIMPER analysis which places the genera with the largest contribution to the dissimilarities that exist between before and during the early recovery. Moreover, *Spirinia* and *Paracomesoma* also contributed to the dissimilarities between before and after the seagrass disappearance. On the other hand, the genus *Terschellingia* showed great contribution to similarities in both times (before and after) indicating that there was no variability in the biomass in the nematodes of this genus. The *Terschellingia* are typically present in poorly oxygenated and organically enriched bottoms, but can also tolerate variations of these conditions and have a well-known tolerance to disturbance (Soetaert et al. 1994; Soetaert et al. 1995; Austen & Somerfield 1997; Schratzberger et al. 2006; Gambi et al. 2008).

The feeding types based on nematode biomass data did not present any significant differences between before and after habitat loss, sites or sampling occasions. The absence of significant differences on the feeding types based on nematode biomass between before the collapse and the early recovery process, demonstrated that although composition of genera has changed, the four trophic groups maintained their proportions. These results were not immediately anticipated since the predators (2B), such as *Sphaerolaimus* and *Metachromadora*, and epistrate feeders (2A), such as

Odontophora, increased their contribution in the nematode biomass. Other authors have reported the increase of epistrate feeders (2A) and predators/omnivores (2B) in unvegetated sediments (Fonseca et al. 2011).

The results of trophic groups based on nematode biomass are in agreement with the results of trophic groups based on nematode density which in turn have also not showed significant differences (Materatski et al., in prep., Chapter II). The lack of differences on feeding groups may be explained by the fact that environmental conditions can still be described as typical intertidal muddy sediments of the estuarine euhaline section, greatly enriched by organic matter, known for generally favouring feeding groups such as non selective deposit feeders (1B) and selective deposit feeders (1A). Additionally the new sediment conditions reflect a strong influence of the marine environment with high salinity and high fractions of sand, silt and clay (Teixeira et al. 2008). Due to these typical intertidal muddy characteristics, the feeding groups such as non selective deposit feeders (1B), usually composed by opportunistic genera (Gallucci et al. 2008; Lee et al. 2001), have also increased their contribution, particularly *Sabatieria* and *Axonolaimus* genera. The genus *Sabatieria* is capable to persist under conditions that are unsuitable for most other nematode species (Tietjen 1980; Hendelberg & Jensen 1993; Steyaert et al. 1999). Once the nematode assemblages density and biomass are closely influenced by changes in sediments – directly through the availability of interstitial habitats, or indirectly through changes in the availability of food and oxygen (McIntyre 1969; Martens & Schockaert 1986; Giere 2009) – species that are clearly adapted to these natural stresses, such as *Paracomesoma*, *Spirinia*, *Terschellingia*, and *Sphaerolaimus*, are the dominant ones.

Important considerations can also be made regarding the nematode morphometric

attributes that showed significant higher values after the collapse in all descriptors, length, width and length/width. A clear trend of larger nematode genera was shown after the collapse, e. g. *Cyartonema* presented a length of 10392 μm and a width of 94 μm (L/W ratio 110), compared to the larger nematodes before the collapse, genus *Linhomoeus*, with a length of 4976 μm and a width of 51 μm (L/W ratio 97). Nematode length values after the collapse are much higher than those observed Romeyn & Bouwman (1983) in the intertidal muddy sediments of Ems-Dollard estuary (England) who reported that the lengths of estuarine nematodes were lower than 5000 μm . Despite being significantly different before and during early recovery, the mean values of nematode length and width do not show such a clear discrepancy. At both ecological conditions (before and after) the mean values of the nematode lengths are less than 2500 μm and greater than 1000 μm , lower than those observed in the deep sea and ocean margins where nematodes were up to 5000 μm long (Soetaert et al. 2002; Soetaert et al. 2009). However, the significant higher values of length registered during the habitat recovery are in accordance to previously results that show that nematodes in sandy sediments are longer in length (Warwick 1971; Heip et al. 1985; Soetaert et al. 2009). In addition, the nematode length may be expected to increase with the increase of the pore size, because nematodes require a surface for propulsion as they move through interstices (Ward et al. 1975). On the other hand, the width mean values at both ecological conditions ranged from 40 μm to 70 μm , these are higher values when compared to Tita et al. (1999) that reported nematode widths ranging from 22.6 to 32.0 μm . Our higher width values may be explained because the range of body width increases with the increase of diversity of the sizes of sediment particles overall (Fleeger et al. 2011). These results suggest that the increase in particle size after the collapse exercised a strong influence on nematode body size,

length and width. The mean L/W ratio of 29 before the collapse is similar to that reported by Soetaert et al. (2002) ($L/W < 29$), a value low because of the characteristics of the study area: organic enrichment, anoxic conditions and low trophic quality. In our case these low L/W values before the collapse can be attributed to the composition of the sediment with high silt and clay percentage that favours stout and slender nematodes which present low L/W ratio (Soetaert et al. 2002). The higher values of L/W ratio 33.4 after the collapse are very similar to those found by Fleege et al. (2011) in sandy sediments and Losi et al. (2013) (L/W 34 and 35, respectively), that suggest a higher L/W of nematodes in disturbed areas in the same sediment depth/layer.

The L/W spectra of three distinct morphological groups showed, before the seagrass disappearance, more prevalence of slender nematodes (L/W ratio of 18-72) (90.4%) followed by stout nematodes (L/W ratio < 18) (5.5%) and long nematodes (L/W ratio > 72) (4.1%). After the collapse the majority of nematodes were also slender (85.2%) followed by long nematodes (9.3%) and stout nematodes (5.4%). These proportions are similar to those observed by Schratzberger et al. (2007) in the southwestern subtidal North Sea, who also recorded that the majority of nematodes were slender (82%), followed by long (12%) and stout (6%).

The main difference on the morphological groups composition, before and after the collapse, lies on the higher percentage of long nematodes after the collapse, which may be the reason for significant differences between both times (before and after). The longer nematodes comprise the genera belonging to the nematodes more adapted to the new ecological conditions, with higher sand values and bare sediments. Nematode body shape has been suggested to be related with the biogeochemical conditions of the sediment as well as with the availability of food (Tita et al. 1999;

Soetaert et al. 2002; Vanaverbeke et al. 2004; Losi et al. 2013). On the other hand, long nematodes are related with a more unstable environment, since their body shape is thought to be advantageous in coarse-sediment habitats (Gerlach 1953; Wieser 1959; Warwick 1971; Tietjen 1976; Thistle & Sherman 1985).

Conclusion

Nematode biomass and morphometric attributes were influenced by the sediment modifications caused by *Z. noltii* disappearance. This was probably due to alterations of sediment grain size conditions such as increased proportions of sand, which are influencing the nematode community, directly through the availability of interstitial habitats as pore spaces, availability of food and oxygen. The information that resulted from biomass, length, width and L/W ratio analyses showed that these parameters are indicative of the functional adaptation of nematodes to the changes of environmental conditions. They have presented some different results from previous ones on the same study area (the Mira estuary) based on abiotic variables and nematode community density analyses, reinforcing the usefulness of nematode biomass and morphometric attributes in the detection and discrimination of different environmental quality status. These parameters have shown a great importance as brief ecological indicators of the health of marine sediments, especially on seagrass, one of the most anthropogenic-affected habitats worldwide, providing complementary types of information different from those based on nematode densities.

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General Conclusion

An integrative approach on the various topics presented in the previous chapters is addressed. This research illustrates the nematode assemblages response to passive natural recovery after a major collapse of seagrass bed *Zostera noltii* on nematode communities in the Mira estuary. *Z. noltii* allows the creation of complex habitats and substrates for several organisms, offering them shelter from predation as well as feeding and nursery areas. Currently, seagrasses are subjected to several threats that represent high economic and biodiversity losses all over the world. Despite that, *Z. noltii* is currently listed as least concern on the International Union for the Conservation of Nature (IUCN) Red List of Threatened Species (2014).

The major collapse of *Z. noltii* and its early recovery process provided a natural study case to ascertain in more detail the processes and relationships between nematode communities and their ecological environment conditions. This study focused on nematodes communities essentially on four perspectives: 1) To investigate the temporal and spatial patterns of nematode community density, taxonomic and functional diversity and genera composition, associated with the early recovery process of the seagrass beds; 2) To investigate the differences of nematode communities, during the stable ecological condition of the seagrass bed before and after the major collapse, during the early recovery process through the analysis of the temporal and spatial distribution of nematode assemblages composition and biodiversity, trophic composition and life strategies; 3) To investigate the differences of biomass, length, width and length/width ratio of nematode communities in the stable ecological condition of the seagrass bed before and during the early recovery process and to discuss these nematodes attributes as indicators of the functional

adaptation of nematodes to the changes of environmental conditions; 4) To use dual isotopes to document food web structure and elucidate the contribution of potential carbon sources to macrofauna and meiofauna diets in *Z. noltii* seagrass beds and in adjacent bare sediments. Additionally, in appendix I it was examined the existence of temporal variation in resource utilization by macrofauna. In appendix II it was examined the validity of mouth-morphology based nematode feeding guilds, based on their trophic position and resource utilization.

The structuring environmental conditions that drive the spatial and temporal variability of the nematode assemblages were very similar between sites in the early recovery process of *Z. noltii*. However, the nematode community density and trophic composition presented significant differences between sites, while the diversity was similar. Contrary to the hypothesis proposed, heterogeneous recovery process of *Z. noltii* with sparse nature and small patches alternating with bare sediment, did not increase the heterogeneity of nematode community distribution between stations. No clear temporal patterns of the nematode density, trophic composition and diversity were observed, despite the features of the seagrass recovery. Small density differences were detected within sampling occasions at each site. The nematode assemblages revealed the ability to withstand the natural variability, providing distinct assemblages typical of the intertidal sediments from euhaline section, naturally adapted to highly stressed conditions, characterized by high densities and diversities.

The differences detected on the environmental conditions between the former data and during the early recovery were highly significant, mainly due to the reduced *Z. noltii* biomass, since seagrass, considered as an ‘ecosystem engineer’, modified significantly the sediment ecological conditions. The differences between the former environmental data and the recovery process demonstrated that nematode density and

genera diversity were influenced by changes of environmental conditions. Before *Z. noltii* disappearance nematode density was consistently higher than after the collapse, while genera diversity showed higher values in the early recovery process, thus indicating that the habitat modifications decreased the nematode density and increased the genera diversity. However, the trophic composition did not show significant differences, demonstrating that the nematode community maintained the same proportions of the four trophic groups, indicating a good ecological state with the typical proportions of feeding types in intertidal seagrass muddy sediment.

The nematode biomass and morphometric attributes were strongly influenced by *Z. noltii* disappearance, probably due to the increase of the sediment grain size conditions with high proportions of sand. The significantly higher values of biomass, length, width and L/W ratio during the habitat recovery indicate that these descriptors could be important ecological indicators of the functional adaptation of nematodes to the changes of environmental conditions. These parameters have shown a great importance as brief ecological indicators of the health of marine sediments, especially on seagrass, one of the most anthropogenic-affected habitats worldwide, providing complementary types of information different from those based only in nematode densities.

Dual isotopes were used to document food web structure and elucidate the contribution of potential carbon sources to macrofauna and meiofauna diets in *Z. noltii* seagrass beds and in adjacent bare sediments. The results obtained were determinant to help explaining nematode assemblages features. The stable isotopic results of estuarine macrobenthos, demonstrated an important evidence that seagrass-associated carbon sources, such as epiphytic microalgae and seagrass detritus, were as important as MPB and SPOM for the diet of these organisms. Moreover, for the first

time, these data confirm the idea that lucinid bivalves host chemoautotrophic sulphur-oxidizing bacteria and obtain a substantial part of their carbon from this symbiosis in estuarine seagrass habitats. Finally, these results have confirmed the direct and indirect importance of seagrass vegetation to the macrobenthos, supporting the idea that carbon inputs associated with seagrass beds in our study area extend well beyond the vegetation boundaries and contribute to the diet of macrobenthos in adjacent sediments. Sequentially, the results of the stable isotope of meiofauna data in seagrass beds at the Mira estuary, suggest that the organic carbon inputs in the meiobenthic food web derive from various resources, namely seagrass detritus, roots, epiphytes, MPB and SPOM, all to some extent being utilized by nematodes and harpacticoid copepods. In addition, chemoautotrophic carbon is also included in the diet of some taxa, like *Terschellingia* most probably via feeding on sulfide-oxidizing bacteria, or such as *Sphaerolaimus* and *Paracomesoma* feeding on preys. The meiofauna results agree with the macrofauna results that “support the idea that carbon inputs associated with seagrass beds extend beyond the vegetation boundaries and contribute to the diet of benthos living adjacent to seagrass vegetation”, including representatives of the predominant meiofaunal taxa. In addition, there is a considerable variation of the resource use and the trophic level found for confamilial or congeneric nematode species, e.g. some unexpected predatory feeding mode was observed in the deposit feeders *Paracomesoma* and the unidentified *Comesomatidae*. For these reasons, in that work it was recommended to combine mouth morphology with stable isotope analysis at the genus or even species level in order to clarify the complex feeding interactions at/near the basis of the benthic food web.

This thesis has contributed to the knowledge of the free-living benthic nematode responses to a habitat recovery process, after an extreme event such as the total

disappearance of *Z. noltii*, due to a comparison with former seagrass bed data. Moreover, this study confirms the importance of biomass and morphometric attributes in analysing nematode responses to environmental changes. The present study incorporates samples from a previous "original" state providing a much broader inventory of the local taxa. The studies contained in this research have not only constructed a robust and useful habitat classification system for seagrass estuaries, but have also demonstrated their use for predicting temporal and spatial differences in highly habitat-specific nematode assemblages. The essence of ecological functioning was maintained after the habitat loss so it is possible to predict that a "good ecological state" can be achieved. In addition, our studies strengthen the use of nematode communities as good ecological indicators and, moreover, they confirm the great impact that seagrasses have on environmental conditions and reinforce the urgent need of public alertness to the importance of the maintenance of these ecosystems.

Future Perspectives

The main objectives of this study were fulfilled, however other questions emerged posing new challenges and defining new research directions. In this section there are presented new research areas drawn from the present work, stressing the importance of further investigation on the processes relating estuarine nematode assemblages and the surrounding estuarine environments.

The results obtained on the structure of nematode community composition during the early recovery process together with the biomass and morphometric attributes, will help in the development of new measures for monitoring estuarine environments strongly threatened around the world.

The improvement of the techniques of extracting living individuals, identifying, handling the nematodes together with the study of nematode features such as biomass and morphometric attributes and how these change under different natural environmental, opens doors to a less explored area that is based on the ecotoxicology of nematode communities.

It would be interesting to find if these nematode indicators such as biomass and morphometric attributes, are influenced by other factors such as imposed toxic effects. By combining the information provided in this study with the toxic pressures in the estuary, valuable insights can be attained for a more quick and adequate management of the estuarine ecosystem.

Appendix I

Vafeiadou, A.-M.; **Materatski, P.**; Adão, H.; De Troch, M.; Moens, T. (2013). Food sources of macrobenthos in an estuarine seagrass habitat (*Zostera noltii*) as revealed by dual stable isotope signatures. *Marine Biology*, 160: 2517-2523.

Food sources of macrobenthos in an estuarine seagrass habitat (*Zostera noltii*) as revealed by dual stable isotope signatures

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Abstract Stable carbon and nitrogen isotope analysis was used to examine the resources and position of macrobenthos in an estuarine seagrass food web in two sampling moments, during summer and winter. The contribution of each food source to the carbon requirements of consumers was estimated by a mixing model. The used carbon sources were largely seagrass associated, although seagrass tissues were utilized by only few species, and equally contributed to microphytobenthos and suspended particulate organic matter. Based on isotopic data, Lucinidae bivalves have an alternative trophic pathway via symbiosis with chemoautotrophic bacteria. Resource utilization inside and adjacent to seagrass beds did not differ significantly, implying that seagrass-associated inputs extend well beyond the borders of the vegetation patches.

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Introduction

Food webs in estuarine ecosystems are characterized by the presence of diverse resources and high macrobenthic diversity (Deegan and Garritt 1997). Macrofauna rely on various carbon sources implying different competitive interactions (Herman et al. 2000) and often exhibit opportunistic feeding behaviour related to changes in habitat and food availability (Deegan and Garritt 1997; Stocks and Grassle 2001).

Seagrass beds contribute to estuarine ecosystem functioning by supporting high biodiversity and more complex food webs than bare sediments (Boström and Mattila 1999). They provide a variety of microhabitats and food, including seagrass leaves and roots, detritus and other associated carbon sources; that is, epiphytes, suspended particulate organic matter (SPOM) trapped in the canopy, and epibenthic and other microalgae in the sediments (Moncreiff and Sullivan 2001).

Although several studies have focused on food utilization by macrobenthos in seagrass beds (Lepoint et al. 2000; Kharlamenko et al. 2001; Moncreiff and Sullivan 2001; Baeta et al. 2009; Carlier et al. 2009; Lebreton et al. 2011; Ouisse et al. 2012), information about the relative importance of resources is still inconclusive (but see Sarà 2006, 2007). This study aims to document food web structure and elucidate the contribution of potential carbon sources to macrofauna diets in an estuarine seagrass habitat, using stable carbon and nitrogen isotopes. We address the following research questions: (1) Do seagrass-associated sources contribute substantially to the diet of macrobenthos? If so, we would expect differences in resource utilization in the seagrass bed vs adjacent unvegetated sediments. (2) Is there temporal variation in resource utilization by macrofauna?

Materials and methods

Sampling area and design

Sampling was located in the Mira estuary, SW Portugal (37°40'N, 8°40'W) (see Fig. 1B in Adão et al. 2009), a small mesotidal system with semidiurnal tides (amplitude 1–3 m during neap and spring tides, respectively). It has a single channel, 5–10 m deep and up to 400 m wide, which allows tidal influence up to 40 km upstream. Due to the limited and seasonally varying freshwater input, the lower section of the estuary has a dominant marine signature.

Sampling was conducted at neap low tide in summer (22/6/2010) and winter (7/2/2011), at two sites (A, ca. 1.5 km from the mouth of the estuary, and B, 2 km more upstream) in intertidal *Zostera noltii* (Hornem.) beds. Samples were collected from two stations (1, 2) at each site, one inside the seagrass bed (A1 and B1), the other in the adjacent bare sediment (A2 and B2). Seagrass vegetation was relatively sparse and significantly less dense (>50 %) in February 2011 than in June 2010.

Sampling and sample treatment

Macrofauna was collected with a core (141 cm²) to 30 cm depth and sieved over a 0.5-mm mesh. The most abundant species were live-sorted, identified and at least three individuals, where possible, were incubated for 4–5 h in filtered habitat water to allow evacuation of gut contents, then stored frozen (−20 °C).

Animals with hard exoskeletons or shells were first dissected. Muscle tissue was used (Yokoyama et al. 2005), except for small organisms which were treated in toto. Samples were divided in two subsamples, one of which was pre-treated with dilute (10 %) HCl to remove carbonates, the other not receiving acid treatment to avoid effects of acidification on $\delta^{15}\text{N}$ (Bunn et al. 1995; Mateo et al. 2008; Vafeiadou et al. in press). All samples were oven-dried (60 °C) and transferred into aluminium cups (6.5 × 8 mm, Elemental Microanalysis Ltd.), pre-combusted for 4 h at 550 °C to remove organic contamination.

Three replicate sediment cores (upper 6 cm) were collected for bulk organic matter analysis. Seagrass was collected by hand and separated in fresh leaves, roots and (partly) decomposed tissue (3 replicates each), then oven-dried (60 °C) for 48 h. Epiphytes were collected by scraping the surface of fresh seagrass leaves with a cover glass. Microphytobenthos (MPB) was collected based on active migration to light through lens tissue (Eaton and Moss 1966). MPB samples were obtained in February and June 2012, 1 year later than, but at very similar sampling moments as the other samples. SPOM was collected by

filtration of 1.5 L of seawater over pre-combusted Whatman GF/F filters. Epiphytic biofilms, MPB and filters were stored frozen prior to further analysis.

Sediments and seagrass materials were first ground to a homogeneous powder. Acidified and non-acidified subsamples were prepared as for macrobenthos. Filters with SPOM were cut in half, one half being acidified for 24 h under HCl vapour, the other not. Epiphytic biofilms and MPB were acidified since insufficient biomass was available for subsampling. All source samples were prepared in pre-combusted silver cups (8 × 5 mm, Elemental Microanalysis Ltd.).

Stable carbon and nitrogen isotope analyses

Isotopic analyses were performed using a ThermoFinnigan Flash 1112 elemental analyser (EA) coupled on-line via a ConFlo III interface to a ThermoFinnigan Delta Plus XL isotope ratio mass spectrometer (IRMS). Stable isotope ratios are expressed relative to the conventional standards in units of parts per thousand, according to the formula:

$$\delta X = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 10^3 \text{ppt}$$

where X is ^{13}C or ^{15}N and R the ratio of $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$. As external lab standards, we used CH-6 (sucrose) and N1 (ammonium sulphate) from the International Atomic Energy Agency, with $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of −10.4 and +0.4 ‰, respectively.

Data analysis

We compared $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ ratios of macrofauna: (1) between vegetated (i.e. A1 and B1) and unvegetated stations (i.e. A2 and B2) and (2) between June and February. Comparisons used paired Student's t tests on pairs of isotopic ratios of the abundant species in both stations and/or months. Normality and homoscedasticity of data were tested using Shapiro–Wilk's and Levene's tests, respectively. No data transformation was required to meet these assumptions. We first assessed whether particular carbon sources had the same δ -values at both sampling times and sites, using the non-parametric Mann–Whitney U test. All statistical analyses were performed using Statistica 7.1 software (StatSoft).

A Bayesian stable isotope mixing model (Parnell et al. 2010) in SIAR 4.1.3 was applied to estimate the contribution of each source to macrobenthos diets. Data were carbon and nitrogen isotopic ratios per replicate sample of each species, excluding Lucinidae sp. based on its much depleted ^{13}C isotopic ratios (see results and discussion), and mean \pm standard deviation (SD) of carbon and nitrogen isotopic ratios per source. Each species was considered at a certain trophic level according to its $\delta^{15}\text{N}$ composition (Vander Zanden and Rasmussen 2001). Mean \pm SD

trophic enrichment factors of 1 ± 1.2 ‰ for $\delta^{13}\text{C}$ and 2.5 ± 2.5 ‰ for $\delta^{15}\text{N}$ were applied for each trophic step (Vander Zanden and Rasmussen 2001; Ouisse et al. 2012). Considering that bulk SOM is a mixture of several sources, we excluded it from the mixing model. To reduce SIAR pitfalls, we ran SIAR several times with different potential sources per consumer, fitting within available knowledge on their feeding mode (Fry 2013). We also ran SIAR to identify the contribution of each carbon source to the SOM pool, with the carbon isotopic ratios of all replicate samples of each source as input data, assuming zero fractionation. Admittedly, isotopic signatures of different SOM components can change during diagenesis (Benner et al. 1991; Altabet 1996) or decomposition (Cloern et al. 2002), as also shown by our data on detrital vs fresh seagrass material. Hence, the isotopic composition of the SOM pool is not a mere reflection of the different inputs. Nevertheless, including it in the SIAR would generally substantially and artificially reduce the estimated contributions of sources with intermediate isotopic signatures.

Results and discussion

Stable isotopic composition of potential food sources and their contributions to the SOM pool

Detritus was more depleted than fresh seagrass. Bulk SOM had $\delta^{13}\text{C}$ values closer to MPB (Fig. 1). As in Ouisse et al. (2012), $\delta^{13}\text{C}$ of epiphytes were intermediate between seagrass detritus and SPOM and very close to MPB signatures (Fig. 1). MPB were also more depleted in ^{13}C than typical for temperate estuarine MPB (Herman et al. 2000; Moens et al. 2002), albeit even lower values have been reported for sandy beach MPB (Maria et al. 2011). SPOM was the most ^{13}C -depleted source with $\delta^{13}\text{C}$ in the range of published values (Baeta et al. 2009). Source isotopic data did not show significant differences between months ($p > 0.05$ in all), except for MPB (for $\delta^{13}\text{C}$: $U = 0$; $Z = -2.7$, $N_1 = 4$, $N_2 = 6$, $p = 0.006$ and for $\delta^{15}\text{N}$: $U = 0$; $Z = 3$, $N_1 = 4$, $N_2 = 6$, $p = 0.003$), in accordance with Baeta et al. (2009) in the Mondego estuary (Portugal). Thus, seagrass detritus had the highest contribution to SOM in June (ca. 0.2–0.3), while epiphytes, MPB and SPOM contributed almost equally (c.a. 0.05–0.2; SM1). In February, the contribution of seagrass detritus decreased (ca. 0–0.1), whereas those of epiphytes and SPOM increased (SM1) as a result of the sparser seagrass vegetation.

Food web analysis of macrobenthos in *Z. noltii* beds

Macrofauna mean $\delta^{13}\text{C}$ ranged from -27.9 to -15.1 ‰ for Lucinidae sp. and Idoteidae sp., respectively, and mean

$\delta^{15}\text{N}$ from -3.1 to 12.6 ‰ for Lucinidae sp. and Glyceridae sp., respectively (Table 1).

We initially performed several SIAR runs, each with different selections of carbon sources for each consumer taking into account available information on its feeding ecology. However, to avoid undue subjectivity and to reduce artefacts (especially biased contributions of sources with intermediate carbon isotopic ratios), we included all candidate sources with the exceptions of SOM (see above) and living seagrass tissue. The latter was included only for specific macrofauna based on their isotopic data (see Table 1) and on the literature. For instance, Idoteidae and *Hydrobia ulvae* (Pennant, 1777) can graze on fresh seagrass leaves (Philippart 1995; Sturaro et al. 2010), while the crab *Carcinus maenas* (Linnaeus, 1758) has a more complex feeding behaviour, including grazing on plant material, predation on other macrofauna, and even cannibalism (Ropes 1968; Moksnes 2004).

Seagrass detritus had a high contribution to the diet of the bivalves *Cerastoderma edule* (Linnaeus, 1758) and *Scrobicularia plana* (da Costa, 1778), the crab *C. maenas*, and the polychaete Nereidae sp. at the vegetated stations in June, with MPB being the second most important source; epiphytes also contributed to the carbon ration of the first two species. Seagrass leaves, detritus and MPB had almost equal contributions to the diet of the gastropod *H. ulvae*. MPB and SPOM were the primary sources for the polychaetes Capitellidae sp. and Cirratulidae sp.1. Results for the unvegetated stations were similar, except for much lower contributions of MPB and SPOM to the diets of *S. plana* and *H. ulvae* (Table 1).

In February, seagrass detritus had a high contribution to the diet of *S. plana* and insect larvae at the unvegetated stations, with epiphytes being their second most important source, while seagrass leaves and detritus contributed importantly to the diet of Idoteidae sp. SPOM was the primary source for all polychaetes at all stations; however, it contributed almost equally with seagrass detritus and epiphytes to the diet of Capitellidae sp. and Nephtyidae sp. (Table 1).

The slightly elevated $\delta^{15}\text{N}$ of *C. maenas* suggests that predation is part of its feeding ecology. Our $\delta^{13}\text{C}$ data point at *S. plana*, Mactridae sp., *H. ulvae*, and the polychaetes Glyceridae sp., Nephtyidae sp. and Nereidae sp. as candidate prey of the crab.

Despite the high nutritional value of epiphytes in temperate seagrass systems (Kitting et al. 1984; Lepoint et al. 2000), their role in *Z. noltii* beds is controversial due to their very low biomass, especially compared to that of benthic microalgae which is much higher in seagrass habitats and more constant throughout the year (Philippart 1995; Lebreton et al. 2009, 2011). Considering the relatively sparse seagrass vegetation in February, we suggest

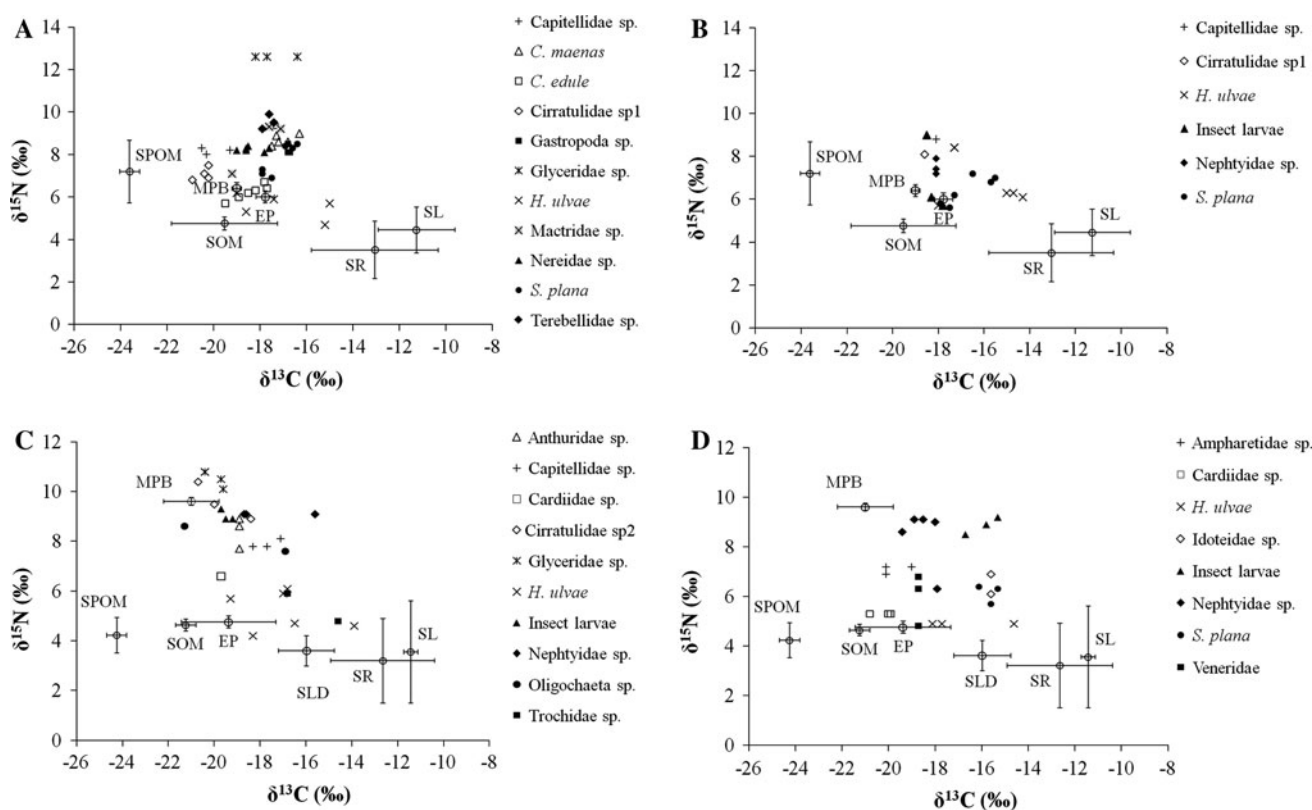


Fig. 1 $\delta^{13}\text{C}/\delta^{15}\text{N}$ plot of macrobenthos and their potential food sources at vegetated and unvegetated stations in June (**a**, **b**) and in February (**c**, **d**), respectively. Data of sources are mean values (\pm SD)

that the SIAR results may in part derive from the similar carbon isotopic ratios of MPB and epiphytes, which may have resulted in an overestimation of the contribution of one source at the expense of the other (Fry 2013).

Microphytobenthos and sedimented phytoplankton are main food sources for epibenthic suspension feeders, grazers and deposit feeders (Herman et al. 2000; Lebreton et al. 2009, 2011). The increased contribution of SPOM—especially for polychaetes and insect larvae—together with its significant contribution to the SOM pool in February, indicates a shift from MPB to SPOM utilization from June to February. $\delta^{15}\text{N}$ placed consumers at four different trophic levels (Table 1). Glyceridae sp. is a top predator; other polychaetes (i.e. Nephtyidae sp., Nereidae sp.), oligochaetes, the crab species and Anthuridae sp. are intermediate predators. SIAR supports the expected suspension-feeding mode for Ampharetidae sp. and Cirratulidae spp. and a deposit-feeding strategy for Terebellidae sp., although their $\delta^{15}\text{N}$ ratios were unexpectedly high.

The isotopic composition of the Lucinidae sp. (mean $\delta^{13}\text{C} = -27.9 \pm 1.2$ ‰ and mean $\delta^{15}\text{N} = -3.1 \pm 1$ ‰) clearly indicates a deviant feeding ecology. The recorded δ -values are in line with those of Lucinidae from mangrove ($\delta^{13}\text{C}$ varying from -32 to -28 ‰ and $\delta^{15}\text{N}$ from -11 to

of all replicates per type of material. (SL, SR and SLD state for seagrass fresh leaves, roots and leaves in decomposition, respectively, EP for epiphytes)

+4 ‰, Bouillon et al. 2008) and deep-sea habitats ($\delta^{13}\text{C} \approx -30$ ‰ and $\delta^{15}\text{N} \approx +4$ ‰, Gulf of Cadiz, Rodrigues et al. 2010). These data confirm the idea that lucinid bivalves host chemoautotrophic sulphur-oxidizing bacteria and obtain a substantial part of their carbon from this symbiosis (Lebata and Primavera 2001; Duperron et al. 2007) and extend this finding for the first time to estuarine seagrass habitats.

Spatial and temporal variation of macrobenthos isotopic composition

There were no significant differences in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of consumers between vegetated and bare stations ($t_{10} = 2.44$, $p = 0.05$ and $t_{10} = 0.56$, $p = 0.585$, respectively) in both months; hence, our results support the idea that carbon inputs associated with seagrass beds in our study area extend well beyond the vegetation boundaries and contribute to the diet of macrobenthos in adjacent sediments (Heck et al. 2008).

Differences in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ between sampling moments were not significant ($t_7 = 1.73$, $p = 0.133$ and $t_7 = 0.09$, $p = 0.926$, respectively), in agreement with Baeta et al. (2009) and Ouisse et al. (2012). While these

Table 1 Mean (\pm SD) carbon and nitrogen isotopic data of macrofauna and relative contributions of potential sources to their carbon requirements per sampling month, at vegetated and unvegetated stations

| Class | Macrofauna species | n | TP | Mean $\delta^{13}\text{C} \pm \text{SD}$ (‰) | Mean $\delta^{15}\text{N} \pm \text{SD}$ (‰) | Relative contribution (CI = 95 %) | | | | |
|---|----------------------------|---|----|--|--|-----------------------------------|-----------|-----------|-----------|-----------|
| | | | | | | SL | SLD | EP | MPB | SPOM |
| <i>June 2010 vegetated stations</i> | | | | | | | | | | |
| Bivalvia | <i>Cerastoderma edule</i> | 6 | 2 | -18.43 ± 0.69 | 6.22 ± 0.34 | Nc | 0.29–0.4 | 0.17–0.33 | 0.24–0.36 | 0.03–0.14 |
| | Lucinidae sp. | 6 | Nc | -28.17 ± 1.07 | -2.92 ± 0.38 | Nc | Nc | Nc | Nc | Nc |
| | Mactridae sp. | 3 | 3 | -17.97 ± 1.1 | 8.53 ± 1.24 | Nc | 0.23–0.37 | 0.16–0.33 | 0.2–0.37 | 0.12–0.28 |
| | <i>Scrobicularia plana</i> | 6 | 3 | -17.2 ± 0.66 | 7.75 ± 0.73 | Nc | 0.31–0.46 | 0.14–0.34 | 0.24–0.37 | 0.01–0.1 |
| Malacostraca | <i>Carcinus maenas</i> | 6 | 3 | -17.07 ± 0.44 | 8.82 ± 0.36 | 0.08–0.19 | 0.18–0.32 | 0.09–0.25 | 0.22–0.34 | 0.12–0.24 |
| Gastropoda | Gastropoda sp. | 3 | 3 | -16.77 ± 0.06 | 8.23 ± 0.23 | Nc | 0.29–0.43 | 0.21–0.39 | 0.15–0.31 | 0.02–0.15 |
| | <i>Hydrobia ulvae</i> | 5 | 2 | -17.04 ± 1.87 | 5.56 ± 0.58 | 0.16–0.3 | 0.18–0.34 | 0.01–0.17 | 0.18–0.32 | 0.01–0.13 |
| Polychaeta | Capitellidae sp. | 3 | 3 | -20.03 ± 0.64 | 8.17 ± 0.15 | Nc | 0.04–0.18 | 0.01–0.16 | 0.28–0.47 | 0.22–0.4 |
| | Cirratulidae sp.1 | 4 | 2 | -20.43 ± 0.33 | 7.08 ± 0.31 | Nc | 0.02–0.1 | 0.01–0.11 | 0.31–0.52 | 0.24–0.46 |
| | Glyceridae sp. | 3 | 4 | -17.43 ± 0.06 | 12.6 ± 0.23 | Nc | 0.17–0.33 | 0.16–0.34 | 0.2–0.36 | 0.19–0.34 |
| | Nereidae sp. | 6 | 3 | -18.35 ± 0.54 | 8.25 ± 0.1 | Nc | 0.24–0.37 | 0.06–0.22 | 0.3–0.46 | 0.17–0.21 |
| | Terebellidae sp. | 3 | 3 | -17.63 ± 0.25 | 9.53 ± 0.35 | Nc | 0.23–0.37 | 0.2–0.36 | 0.18–0.33 | 0.13–0.28 |
| <i>June 2010 unvegetated stations</i> | | | | | | | | | | |
| Bivalvia | Lucinidae sp. | 6 | Nc | -27.85 ± 0.56 | -3.35 ± 0.81 | Nc | Nc | Nc | Nc | Nc |
| | <i>Scrobicularia plana</i> | 6 | 2 | -16.73 ± 0.99 | 6.43 ± 0.66 | Nc | 0.32–0.49 | 0.17–0.27 | 0.15–0.3 | 0–0.1 |
| Gastropoda | <i>Hydrobia ulvae</i> | 6 | 2 | -16.1 ± 1.61 | 6.87 ± 1.21 | 0.22–0.32 | 0.18–0.32 | 0.1–0.27 | 0.1–0.25 | 0.02–0.14 |
| Insecta | Insect larvae | 3 | 2 | -18.2 ± 0.36 | 6.93 ± 1.8 | Nc | 0.19–0.35 | 0.17–0.35 | 0.2–0.37 | 0.16–0.32 |
| Polychaeta | Capitellidae sp. | 1 | 3 | -18.1 | 8.8 | Nc | 0.17–0.27 | 0.16–0.26 | 0.16–0.26 | 0.16–0.25 |
| | Cirratulidae sp.1 | 1 | 3 | -18.6 | 8.1 | Nc | 0.17–0.36 | 0.16–0.35 | 0.17–0.38 | 0.16–0.38 |
| | Nephtyidae sp. | 3 | 3 | -18.13 ± 0.06 | 7.5 ± 0.36 | Nc | 0.21–0.36 | 0.15–0.35 | 0.22–0.38 | 0.13–0.31 |
| <i>February 2011 vegetated stations</i> | | | | | | | | | | |
| Bivalvia | Cardiidae sp. | 1 | 2 | -19.7 | 6.6 | Nc | 0.17–0.36 | 0.16–0.36 | 0.15–0.35 | 0.18–0.37 |
| | Lucinidae sp. | 3 | Nc | -28.37 ± 1.63 | -1.67 ± 0.42 | Nc | Nc | Nc | Nc | Nc |
| Malacostraca | Anthuridae sp. | 3 | 3 | -18.87 ± 0.06 | 8.4 ± 0.62 | Nc | 0.18–0.34 | 0.17–0.36 | 0.01–0.17 | 0.28–0.41 |
| Gastropoda | <i>Hydrobia ulvae</i> | 6 | 2 | -16.97 ± 1.84 | 5.2 ± 0.79 | 0.19–0.32 | 0.17–0.35 | 0.12–0.3 | 0–0.05 | 0.19–0.34 |
| | Trochidae sp. | 2 | 2 | -15.7 ± 1.56 | 5.35 ± 0.78 | 0.2–0.35 | 0.17–0.33 | 0.13–0.3 | 0.01–0.18 | 0.06–0.24 |
| Insecta | Insect larvae | 3 | 3 | -19.47 ± 0.25 | 9.03 ± 0.23 | Nc | 0.12–0.27 | 0.17–0.36 | 0.04–0.22 | 0.3–0.44 |
| Oligochaeta | Oligochaeta sp. | 2 | 3 | -19.1 ± 3.11 | 8.1 ± 0.71 | Nc | 0.17–0.37 | 0.17–0.38 | 0.02–0.24 | 0.21–0.4 |
| Polychaeta | Capitellidae sp. | 3 | 3 | -17.7 ± 0.6 | 7.9 ± 0.17 | Nc | 0.25–0.4 | 0.17–0.36 | 0.1–0.15 | 0.2–0.35 |
| | Cirratulidae sp.2 | 3 | 3 | -19.7 ± 1.18 | 9.6 ± 0.75 | Nc | 0.01–0.16 | 0.1–0.33 | 0–0.18 | 0.3–0.51 |
| | Glyceridae sp. | 4 | 4 | -20.03 ± 0.43 | 10.55 ± 0.33 | Nc | 0–0.1 | 0.02–0.2 | 0–0.1 | 0.5–0.69 |
| | Nephtyidae sp. | 3 | 3 | -17.63 ± 1.76 | 9.07 ± 0.06 | Nc | 0.24–0.38 | 0.2–0.37 | 0.03–0.19 | 0.2–0.36 |
| <i>February 2011 unvegetated stations</i> | | | | | | | | | | |
| Bivalvia | Cardiidae sp. | 3 | 2 | -20.23 ± 0.49 | 5.33 ± 0.06 | Nc | 0.14–0.29 | 0.16–0.35 | 0.01–0.17 | 0.28–0.44 |
| | Lucinidae sp. | 3 | Nc | -26.7 ± 1.01 | -4.43 ± 0.35 | Nc | Nc | Nc | Nc | Nc |
| | <i>Scrobicularia plana</i> | 3 | 2 | -15.67 ± 0.4 | 6.13 ± 0.38 | Nc | 0.36–0.54 | 0.17–0.38 | 0–0.15 | 0.01–0.14 |
| | Veneridae sp. | 3 | 2 | -18.7 ± 0.1 | 5.97 ± 1.04 | Nc | 0.25–0.38 | 0.17–0.36 | 0.02–0.16 | 0.2–0.35 |
| Malacostraca | Idoteidae sp. | 2 | 2 | -15.6 | 6.5 ± 0.57 | 0.21–0.35 | 0.17–0.32 | 0.12–0.27 | 0.03–0.18 | 0.03–0.18 |
| Gastropoda | <i>Hydrobia ulvae</i> | 3 | 2 | -16.80 ± 1.92 | 4.8 ± 0.1 | 0.18–0.31 | 0.16–0.33 | 0.13–0.3 | 0–0.12 | 0.14–0.28 |

Table 1 continued

| Class | Macrofauna species | n | TP | Mean $\delta^{13}\text{C} \pm \text{SD}$ (‰) | Mean $\delta^{15}\text{N} \pm \text{SD}$ (‰) | Relative contribution (CI = 95 %) | | | | |
|------------|--------------------|---|----|--|--|-----------------------------------|-----------|-----------|-----------|-----------|
| | | | | | | SL | SLD | EP | MPB | SPOM |
| Insecta | Insect larvae | 3 | 3 | -15.93 ± 0.71 | 8.87 ± 0.35 | Nc | 0.32–0.5 | 0.2–0.39 | 0.02–0.15 | 0.03–0.17 |
| Polychaeta | Ampharetidae sp. | 3 | 2 | -19.73 ± 0.64 | 7.1 ± 0.17 | Nc | 0.05–0.2 | 0.15–0.35 | 0.01–0.21 | 0.3–0.46 |
| | Nephtyidae sp. | 5 | 3 | -18.54 ± 0.63 | 8.42 ± 1.2 | Nc | 0.22–0.35 | 0.16–0.34 | 0.01–0.12 | 0.3–0.42 |

n number of replicate samples, TP Trophic position, CI confidence intervals, Nc not considered

results demonstrate that macrobenthos in *Z. noltii* beds in southern Europe rely on the same food sources year-round, this does not necessarily imply constant contributions of different carbon sources across seasons. Indeed, our results showed that the contribution of carbon sources differed between sampling times for some species.

Conclusions

In spite of the relatively large variation of stable isotopic ratios for most macrofauna, there is evidence that seagrass-associated carbon sources, such as epiphytic microalgae and seagrass detritus, were equally important as MPB and SPOM for the diet of estuarine macrobenthos. Resource utilization inside and adjacent to seagrass patches was very similar across species, demonstrating that seagrass-associated inputs extend beyond the borders of the vegetation patches. This highlights the importance, direct and indirect, of seagrass vegetation to the macrobenthos. Furthermore, this study confirms for the first time the dependence of Lucinidae on symbiotic chemoautotrophic bacteria in estuarine seagrass environments.

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Appendix II

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Resource utilization and trophic position of nematodes and harpacticoid copepods in and adjacent to *Zostera noltii* beds

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Abstract. This study examines the resource use and trophic position of nematodes and harpacticoid copepods at the genus/species level in an estuarine food web in *Zostera noltii* beds and in adjacent bare sediments using the natural abundance of stable carbon and nitrogen isotopes. Microphytobenthos and/or epiphytes are among the main resources of most taxa, but seagrass detritus and sediment particulate organic matter contribute as well to meiobenthos nutrition, which are also available in deeper sediment layers and in unvegetated patches close to seagrass beds. A predominant dependence on chemoautotrophic bacteria was demonstrated for the nematode genus *Terschellingia* and the copepod family Cletodidae. A predatory feeding mode is illustrated for *Paracomesoma* and other Comesomatidae, which were previously considered first-level consumers (deposit feeders) according to their buccal morphology. The considerable variation found in both resource use and trophic level among nematode genera from the same feeding type, and even among congeneric nematode species, shows that the interpretation of nematode feeding ecology based purely on mouth morphology should be avoided.

well as critical food resources and habitats for many others (Walker et al., 2001). Seagrass beds typically support higher biodiversity and faunal abundance compared to the adjacent unvegetated areas (Edgar et al., 1994) due to both increased food supply and reduced predation risks (Heck et al., 1989; Ferrell and Bell, 1991). Furthermore, they strongly influence the associated fauna by modifying hydrodynamics (Fonseca and Fisher, 1986) and by altering the energy flux either directly, through release of dissolved organic carbon into the water column, or indirectly, by contributing to the detritus pool after decomposition (Boström and Bonsdorff, 1997).

Several studies during the last decade have used natural stable isotope ratios to elucidate the principal food sources of macrobenthos in seagrass beds, stressing the importance of seagrass-associated sources and/or microphytobenthos (MPB) (Lepoint et al., 2000; Kharlamenko et al., 2001; Moncreiff and Sullivan, 2001; Baeta et al., 2009; Carlier et al., 2009; Lebreton et al., 2011; Ouisse et al., 2012; Vafeiadou et al., 2013a). Less information is available for meiobenthos resource utilization in seagrass beds (Vizzini et al., 2000b, 2002a; Baeta et al., 2009; Leduc et al., 2009; Lebreton et al., 2011, 2012), with none of the studies including meiofauna at the level of feeding types, families, genera or species. The few studies using natural isotope abundances to unravel food resources of coastal meiofauna at this level (Carman and Fry, 2002; Moens et al., 2002, 2005, 2013; Rzeznik-Orignac et al., 2008) do not examine seagrass habitats.

1 Introduction

Seagrass meadows form unique, productive and highly diverse ecosystems throughout the world (Hemminga and Duarte, 2000). They stabilize and enrich sediments, and provide breeding and nursery grounds for various organisms as

The present study aims to assess the principal carbon resources of the nematode and harpacticoid copepod assemblages, at the species, genus and family level, in *Zostera noltii* Hornem. seagrass beds and in adjacent bare sediments. In light of several stable isotope studies which have stressed the predominant role of MPB as a carbon resource to intertidal meiofauna (Moens et al., 2002, 2005; Rzeznik-Orignac et al., 2008; Maria et al., 2012), we hypothesized that MPB would be the principal carbon resource for the majority of taxa in bare sediments. In vegetated sediments, seagrass-associated resources (i.e. seagrass detritus and epiphytes) could also contribute, and higher sedimentation rates would likely raise the contribution of suspended particulate organic matter (SPOM) to meiofauna diets, much like in salt marshes (Moens et al., 2005). We also expected MPB and SPOM to contribute more at the sediment surface than deeper down in the sediment given Rudnick's theory (1989) which proposed a different resource utilization by meiofauna in the sediment surface than in deeper layers: fresh phytodetritus would be the principal resource for nematodes living in the upper 2 cm of the sediment, whereas deeper down, nematodes would mainly feed on larger fractions of buried, more refractory detritus. Thus, we would expect a higher contribution of detrital organic matter than of MPB or SPOM in deeper sediment layers.

So far, nematodes have been classified in feeding guilds based on buccal morphology (Wieser, 1953; Jensen, 1987; Moens and Vincx, 1997). Nevertheless, stable isotope data and in situ observations of living nematodes have shown that such stoma-morphology based guild classifications do not always provide good predictions of nematode resource utilization and even trophic level (Moens et al., 2005). In harpacticoid copepods, there is also no straightforward link between the morphology of the mouth parts and their food resources (Hicks and Coull, 1983; De Troch et al., 2006). Therefore, we also examined the validity of existing mouth-morphology based nematode feeding guilds, based on their trophic position and resource utilization as revealed by the stable isotope data obtained in this study. If current guild classifications represent real functional groupings, then resource utilization and trophic level within feeding guilds should be very similar, while it would differ between guilds.

2 Materials and methods

2.1 Study area and sampling design

Sampling was conducted at the Mira estuary (37°40' N, 8°40' W, SW Portugal), a small mesotidal system with a semidiurnal tidal regime (amplitude of 1–3 m during neap and spring tides, respectively). It has a single channel, 5–10 m deep and up to 400 m wide, which allows tidal influence to extend 40 km upstream. Together with the Mira River and its surrounding intertidal area it is included in the pro-

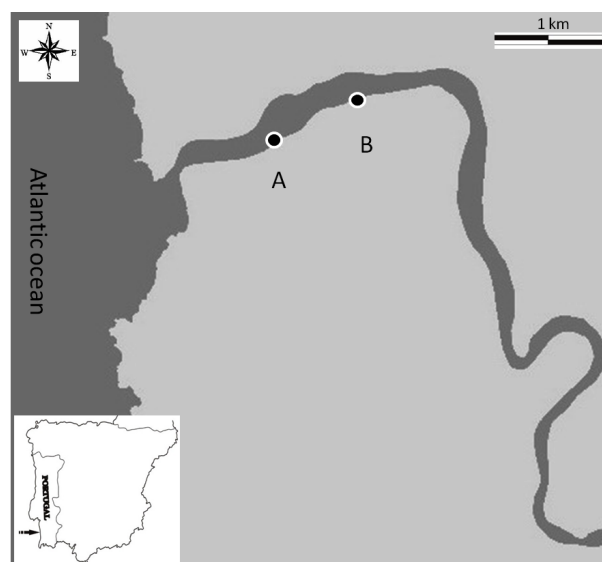


Figure 1. Map of the study area: Mira estuary (Portugal) and sampling sites (A and B).

tected Sudoeste Alentejano e Costa Vicentina natural park (Fig. 1). This estuary is considered relatively undisturbed and free from industrial pollution (Costa et al., 2001). Our study area was located at two sites of the intertidal area at the lower section, ca. 1.5 km from the mouth of the estuary (i.e. sampling site A) and ca. 2 km further upstream (i.e. sampling site B). Due to the low, seasonal and limited freshwater input, the lower section of the estuary has a significant marine signature. In both sites, sediments were sparsely covered with *Zostera noltii*; seagrass vegetation was less dense (ca 50 % difference) in February 2011 than in June 2010 (Vafeiadou et al., 2013a). These seagrass beds used to be denser in the past, but the vegetation is under recovery after a major collapse in 2008 (Adão et al., 2009; Cunha et al., 2013). Samples were collected on two instances (22 June 2010 and 7 February 2011), during low tide (tidal amplitude of 3 m). We sampled two random stations at each sampling site (i.e. A and B), one located inside the seagrass vegetation (i.e. A1 and B1) and the other in adjacent bare sediments (i.e. A2 and B2).

2.2 Sampling of carbon resources and meiobenthos

Fresh seagrass leaves, roots and seagrass detrital material were collected randomly from each vegetated station (i.e. A1 and B1), thoroughly rinsed and carefully scraped off using a cover glass to remove epiphytes, which were collected separately. To obtain bulk sediment organic matter we sampled three replicate cores (10 cm²) of the upper 6 cm of sediment from all stations. The epipellic fraction of MPB was collected via migration through the lens tissue method (Eaton and Moss, 1966) 1 year later than the other samples, but at very similar sampling times and conditions (February and June 2012) because samples collected during the 2010/2011

campaigns yielded insufficient MPB biomass for reliable nitrogen isotopic analysis. 1.5 L of seawater was filtered over pre-combusted Whatman GF/F filters to collect SPOM. Seagrass material and bulk sediment samples were oven dried (60 °C) for 48 h before preservation and stored in desiccators; all other samples were stored frozen.

Meiobenthos samples were obtained by forcing hand cores (10 cm²) to a depth of 6 cm into the sediment at all stations. Each sediment sample was divided into three depth layers: 0–2, 2–4 and 4–6 cm. Seven replicate samples were randomly collected from each station within a 100 m² area and then pooled into one bulk sample considered representative for a particular station. Pooling of replicate samples was done to ensure that enough biomass of several genera/species could be obtained for the stable isotope analyses. Meiobenthos samples were stored frozen prior to elutriation and analysis.

2.3 Preparation of samples for stable isotope analyses

Dried seagrass and bulk sediment samples were homogenised, weighed (0.3–0.7 mg dry weight of seagrass, 20–60 mg dry weight of sediment) and transferred into silver cups (8 × 5 mm, Elemental Microanalysis Ltd) which had been pre-treated for 4 h at 550 °C to remove organic contamination. Two subsamples were then prepared: the first was acidified with dilute HCl to remove carbonates, the second was not acidified, to eliminate any effects of acidification on nitrogen isotopic signatures (Vafeiadou et al., 2013b). A drop of milli-Q water was added to acidified samples which then were oven dried (60 °C) for 48 h. Epiphyte and MPB samples were all acidified since insufficient biomass was available for subsampling. The Whatman GF/F filters were divided in two; only one half was acidified under HCl vapour for 24 h, the other not. All samples were prepared in pre-combusted silver cups.

Meiofauna was elutriated using density centrifugation in Ludox HS40 colloidal silica, which does not affect isotope signatures (Moens et al., 2002). No other chemicals were used during processing of the meiofauna samples. The most abundant nematode and copepod taxa were hand-sorted and identified at the genus or family level under a stereomicroscope. Individuals were hand-picked with a fine needle, rinsed several times in milli-Q water to remove adhering particles, and finally transferred into a drop of milli-Q water in pre-combusted aluminium cups (6 × 2.5 mm, Elemental Microanalysis Ltd). The number of specimens transferred into the cups depended on the abundance and individual biomass of the different taxa. We aimed at a sample mass > 5 µg for the element of interest, be it C, N or both. Thus, 10–40 individuals were pooled together for a copepod sample and 10–300 for a nematode sample, depending on their size. In many cases though, the biomass of the sample was sufficient only for reliable carbon analysis but not for nitrogen analysis. Thus, despite the combined $\delta^{13}\text{C}$ / $\delta^{15}\text{N}$ analysis per sample, we finally obtained different sample numbers for the $\delta^{13}\text{C}$

and $\delta^{15}\text{N}$ data. Because of very low meiofauna abundances below a depth of 2 cm, for most taxa at this depth we obtained sufficient biomass for only a single isotope measurement.

2.4 Stable isotope analyses

Isotopic analyses of resources and meiofauna were performed using a ThermoFinnigan Flash 1112 elemental analyser (EA) coupled online via a ConFlo III interface to a ThermoFinnigan Delta Plus XL isotope ratio mass spectrometer (IRMS), with analytical reproducibility typically ≤ 0.2 ‰ for both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. All resource samples were measured in He-dilution mode, except for the epiphyte samples. These, as well as all meiofauna samples, were measured without He-dilution. Stable isotope ratios are expressed in units of parts per thousand, according to the formula:

$$\delta X = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 10^3,$$

where X is ^{13}C or ^{15}N and R the ratio of $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$. As external lab standards, we used CH-6 (sucrose) and N1 (ammonium sulfate) from the International Atomic Energy Agency, with $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of -10.4 and $+0.4$ ‰, respectively.

When measuring samples containing limited biomass, caution is needed when assessing the results of IRMS. Based on prior tests with decreasing mass of standards of known isotopic ratios, we discarded all results of samples yielding amplitudes smaller than 200 mV. We measured external standards for linear corrections of small analytical errors in the obtained δ -values. Further, we routinely corrected the obtained sample δ -values for the contribution of blanks using the formula:

$$\delta_{\text{organic matter}} = (\delta_{\text{sample}} \times \text{amplitude}_{\text{sample}} - \delta_{\text{blank}} \times \text{amplitude}_{\text{blank}}) / \text{amplitude}_{\text{organic matter}},$$

where $\delta_{\text{organic matter}}$ is the real δ -value of the material of interest and $\text{amplitude}_{\text{organic matter}} = \text{amplitude}_{\text{sample}} - \text{amplitude}_{\text{blank}}$. Such “blank correction” is important in samples with low amplitudes, where even small blanks may contribute significantly to the measured δ_{sample} (Moens et al., 2013).

2.5 Data analysis

For the interpretation of stable isotope data and for mixing model computations, trophic enrichment factors of 1 ± 1.2 ‰ for $\delta^{13}\text{C}$ and 2.5 ± 2.5 ‰ for $\delta^{15}\text{N}$ were adopted for each trophic step (Vander Zanden and Rasmussen, 2001). The comparison of stable isotope data of meiobenthos between vegetated and bare sediments was performed using paired Student's t tests. For this comparison we used only $\delta^{13}\text{C}$ data of those taxa which occurred in the upper 2 cm of both types of sediments. Data from deeper layers and of $\delta^{15}\text{N}$ were not included in this analysis because of a lack of sufficient replication. No data transformation was applied since

the assumptions of normality and homoscedasticity (tested by Cochran's test) were met. The validity of the comparison, with type of sediment as the only factor, was based on the fact that resource isotope signatures did not differ across months or stations (Vafeiadou et al., 2013a). These univariate statistical analyses were performed using Statistica 6 software (StatSoft).

The Bayesian stable isotope mixing model MixSIR (Semmens and Moore, 2008; MixSIR Version 1.0.4. for MATLAB, R2013a, The MathWorks) was applied to the present data, to calculate the relative contributions of potential food resources to the diets of meiofauna. We used the following input data for consumers: $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of each replicate sample separately per taxon, only including data of those samples for which we obtained both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. Input data for potential resources were: mean and SD of $\delta^{13}\text{C}$ and of $\delta^{15}\text{N}$ of all replicate samples per resource. Seagrass leaves were excluded from the model because meiofauna are unlikely to graze directly on living seagrass tissue, both because of the limited direct physical contact between endobenthic meiofauna and living seagrass leaves and because of the absence of any reports showing that meiofauna can graze on living macrophyte tissue. Seagrass detritus, however, was included as a candidate resource; it is unclear whether meiofauna can directly utilize macrophyte detritus, but they are certainly capable of grazing on micro-organisms which decompose the detritus (Moens and Vincx, 1997; Cnudde et al., 2013) and which may have almost identical carbon isotope ratios (Boschker et al., 1999). Seagrass roots were also considered as a potential resource; although they might not be directly grazed upon by meiofauna, they may indirectly contribute to the food web via root exudates consumed by microbiota, even though this link was not detectable in a study on *Zostera marina* (Boschker et al., 2000). Seagrass roots and detritus were pooled as one "seagrass resource" by calculating the mean and SD of their isotopic signatures. We did the same for epiphytes and MPB. In both cases, isotopic ratios of both resources strongly overlapped, hampering adequate assignment of the contribution of each resource separately by the mixing model. A higher number of potential resources also bears upon the performance of the isotope mixing model (Parnell et al., 2010; Middelburg, 2014). Although not measured here, chemoautotrophic bacteria were added as an additional resource based on the $\delta^{13}\text{C}$ obtained here for the nematode genera *Terschellingia* and *Sabatieria* and for the copepod family Cletodidae and on literature information (see the discussion); we adopted an average $\delta^{13}\text{C}$ of $-35 \pm 5\text{‰}$ for this resource (based on data for sulfide-oxidizing bacteria in Robinson and Cavanaugh, 1995) and an average $\delta^{15}\text{N}$ of $4 \pm 0.5\text{‰}$, based on our own data for the three aforementioned taxa, since we found no information on the $\delta^{15}\text{N}$ of sulfide-oxidizing bacteria in the literature. We ran MixSIR with 10 000 iterations, without resource contribution data defined a priori. The model was applied separately for

seagrass beds and bare sediments, and for the surface and deeper sediment layers.

3 Results

3.1 Stable isotope signatures of meiobenthos

Overall, the present study includes $\delta^{13}\text{C}$ data of 20 nematode taxa, 16 of which were identified to the genus level (two genera were represented by two species each) and two to the family level (unidentified Comesomatidae and Chromadoridae), as well as four harpacticoid copepod families (Canuellidae, represented here by the genus *Sunaristes*, Cletodidae, Ectinosomatidae and Harpacticidae, this last taxon being present only in deeper sediments) (Tables 1 and 2). The $\delta^{15}\text{N}$ data are available for 8 of the 16 nematode genera and the unidentified Comesomatidae, and for two copepod families (Canuellidae and Cletodidae) (Tables 1 and 2). Although this data set includes most of the abundant genera of this nematode assemblage (Table 1), some abundant genera are not represented here because of their low individual biomass, hampering the collection of sufficient biomass for stable isotope analysis.

The $\delta^{13}\text{C}$ of most meiofauna from the upper 2 cm ranged from $-22.7 \pm 1.2\text{‰}$ (*Spirinia parasitifera*) to -11.9‰ (*Theristus*) (Fig. 2a), and $\delta^{15}\text{N}$ ranged from 3.9‰ (*Sunaristes*) to 10.8‰ (Comesomatidae) (Fig. 2b). The nematode genus *Terschellingia* and the copepod family Cletodidae had much lower $\delta^{13}\text{C}$ (mean $\delta^{13}\text{C} \pm \text{SD} = -41.7 \pm 2.4\text{‰}$ and $-33.2 \pm 5.5\text{‰}$, respectively; Fig. 2a) compared to all other meiofauna. *Terschellingia* also had very low $\delta^{15}\text{N}$ values ($2.8 \pm 1.9\text{‰}$; Fig. 2b). Most taxa had $\delta^{13}\text{C}$ in the range of MPB and epiphytes, whereas *Spirinia parasitifera* and *Sabatieria* sp. 2 were more depleted in ^{13}C , with $\delta^{13}\text{C}$ values close to SPOM ($-24.1 \pm 1.2\text{‰}$; Figs. 2a and 3). *Daptonema*, *Metachromadora*, *Spirinia* sp. 2, *Ptycholaimellus* and *Theristus* were comparatively enriched in ^{13}C , with values close to those of seagrass detritus ($-16.0 \pm 1.1\text{‰}$; Figs. 2a and 3). The comparison of $\delta^{13}\text{C}$ of meiobenthos from the surface sediment layers between vegetated and bare sediments did not reveal any significant differences ($df = 32$, $t = 1.35$; $p > 0.05$). The $\delta^{15}\text{N}$ data clearly show the presence of more than one trophic level in this nematode assemblage in the upper 2 cm, with *Sphaerolaimus*, *Paracomescuma* and unidentified Comesomatidae belonging to a higher trophic level than all other meiofauna (Figs. 2b and 3).

The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ data from the deeper sediment layers (2–6 cm) are available for a lower number of nematode and copepod taxa due to the overall low meiofauna abundances in these deeper layers (Table 2). Most $\delta^{13}\text{C}$ ranged from -29.8‰ (*Paracanthochus*) to $-14.4 \pm 0.4\text{‰}$ (*Metachromadora*), with the exception of *Terschellingia* and Cletodidae, which had much lower $\delta^{13}\text{C}$ ($-40.4 \pm 4.5\text{‰}$ and -33.5‰ , respectively; Table 2). The $\delta^{13}\text{C}$ of most taxa in

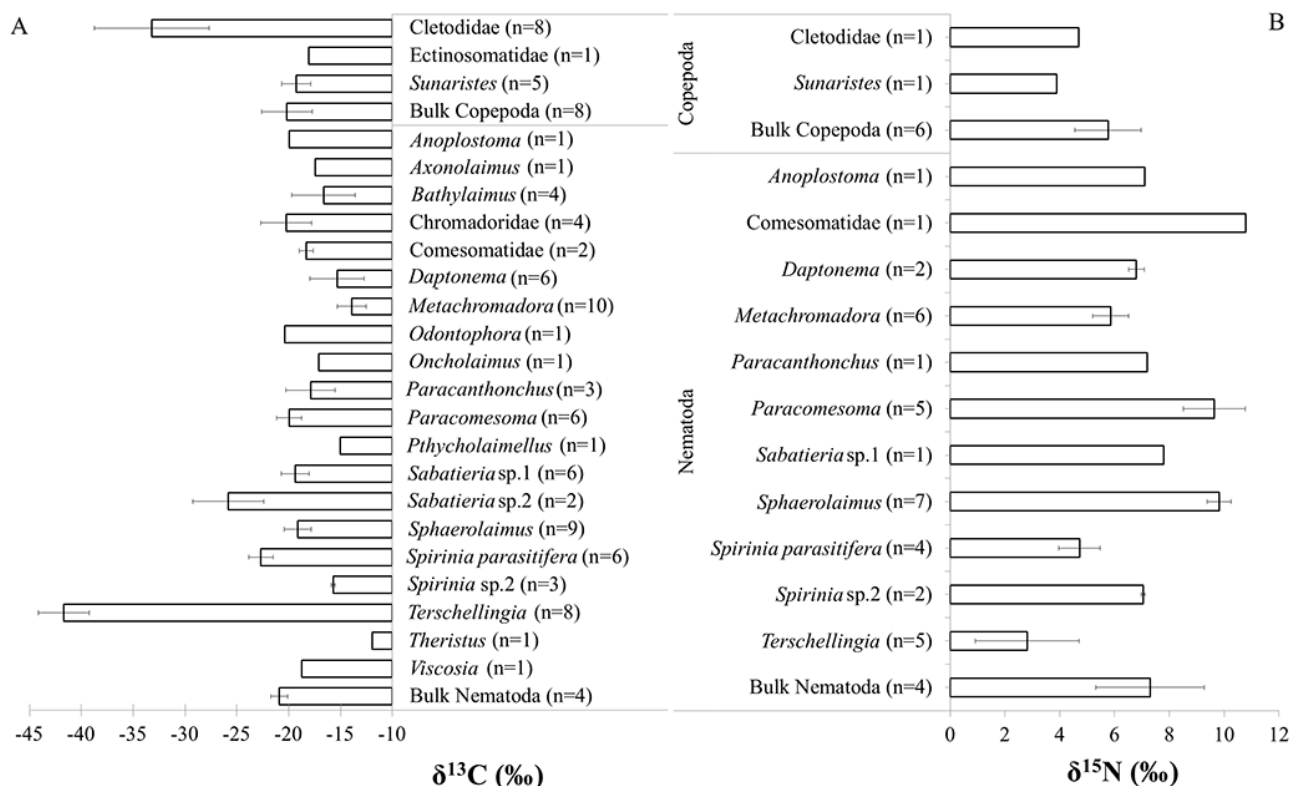


Figure 2. Stable carbon (A) and nitrogen (B) isotope signatures of meiobenthos from the upper 2 cm. Data are mean values (\pm SD) of all replicate samples per taxon, from both vegetated and bare stations (n = number of replicate samples).

the deeper sediment layers (i.e. *Anoplostoma*, *Bathylaimus*, *Oncholaimus*, *Paracanthionchus*, *Sphaerolaimus* and *Spirinia parasitifera*) were more ^{13}C -depleted (by > 4 ‰), and closer to those of SPOM than those from the same taxa at the sediment surface, where they had more intermediate values. The $\delta^{13}\text{C}$ of *Oncholaimus*, *Paracanthionchus*, *Sabatieria* sp. 2, *Spirinia parasitifera* and Harpacticidae were even more depleted than any other measured resource. In contrast, $\delta^{13}\text{C}$ of the nematodes *Daptonema*, *Metachromadora* and *Spirinia* sp. 2 and of the copepod genus *Sunaristes* were in the range of values for seagrass detritus, epiphytes and/or MPB (Table 2).

3.2 Application of the isotope mixing model MixSIR

Applying the isotope mixing model MixSIR to our data yielded model estimations of the proportional contributions of each resource to the diet of each nematode genus/species or copepod family/genus, in seagrass vegetated and bare sediments, and in surface and deeper layers (Table 3). Seagrass-derived carbon (detritus and/or roots) contributed more than other resources to the requirements of *Metachromadora*: 0.75 (0.60–0.88) in seagrass beds and 0.85 (0.70–0.95) in bare sediments, as well as of *Daptonema* with contributions of 0.70 (0.48–0.87) and 0.71 (0.31–0.89) and of *Spirinia* sp. 2 with contributions of 0.59 (0.28–0.81) and 0.67 (0.25–0.86),

in seagrass beds and in bare sediments, respectively (proportional contributions per unit are given as median and lower to upper limits of 95 % confidence intervals; Table 3). Suspended particulate organic matter contributed predominantly to the requirements of *Sphaerolaimus*: 0.27 (0.02–0.75) and 0.20 (0.01–0.72), of *Paracomesoma*: 0.29 (0.03–0.73) and 0.39 (0.02–0.78) and of *Spirinia parasitifera*: 0.37 (0.25–0.78) and 0.34 (0.02–0.76), in seagrass beds and in bare sediments, respectively (Table 3); nevertheless, seagrass resources and chemoautotrophic bacteria also contributed substantially to the diet of the aforementioned taxa. The very wide range of contributions covered by the 95 % confidence intervals is largely a result of including the very ^{13}C -depleted chemoautotrophic bacteria as a candidate resource. In addition, if chemoautotrophic bacteria are not included as a resource in the model, the contribution of SPOM to the diets of several taxa substantially increases. For example, running MixSIR for the three taxa above without chemoautotrophic bacteria as a potential resource yielded SPOM contributions for *Sphaerolaimus*, *Paracomesoma* and *Spirinia parasitifera* of 0.77 (0.64–0.89), 0.80 (0.66–0.92) and 0.83 (0.69–0.94), respectively, in seagrass vegetation, and of 0.67 (0.50–0.83), 0.72 (0.57–0.86) and 0.79 (0.65–0.93), respectively, in bare sediments. Microphytobenthos and/or epiphytes contributed substantially to the diet of most nematode and copepod

Table 1. Relative abundance (%) of nematode genera in *Zostera noltii* beds and stable isotope data of the potential carbon resources and meiofauna from the upper 2 cm in seagrass beds and bare sediments (n = number of replicate samples).

| Meiofauna | Rel. abundance (%) | | | Mean $\delta^{13}\text{C} \pm \text{SD}$ (‰) | | | | Mean $\delta^{15}\text{N} \pm \text{SD}$ (‰) | | | |
|---|--------------------|-------|-----|--|-----|----------------|-----|--|-----|----------------|-----------|
| | June | Feb | n | Seagrass beds | n | Bare sediments | n | Seagrass beds | n | Bare sediments | |
| <i>NEMATODA</i> | | | | | | | | | | | |
| <i>Anoplostoma</i> | 0.69 | 0.74 | 1 | −19.9 | | | 1 | 7.1 | | | |
| <i>Axonolaimus</i> | 1.25 | 5.10 | | | 1 | −17.4 | | | | | |
| <i>Bathylaimus</i> | 0.29 | 0.35 | 3 | −16.7 ± 3.7 | 1 | −16.5 | | | | | |
| Chromadoridae | | | 4 | −20.2 ± 2.5 | | | | | | | |
| Comesomatidae | | | 2 | −18.3 | | | 1 | 10.8 | | | |
| <i>Daptonema</i> | 3.78 | 7.71 | 2 | −15.7 | 4 | −15.1 ± 2.8 | 1 | 7 | 1 | | 6.6 |
| <i>Metachromadora</i> | 2.29 | 4.37 | 5 | −13.9 ± 1.6 | 5 | −13.8 ± 1.5 | 4 | 5.9 ± 0.8 | 2 | | 5.8 |
| <i>Odontophora</i> | 8.53 | 6.61 | | | 1 | −20.4 | | | | | |
| <i>Oncholaimus</i> | 1.96 | 0.59 | 1 | −17.1 | | | | | | | |
| <i>Paracanthochus</i> | 0.33 | 0.01 | 2 | −17.2 | 1 | −19.2 | 1 | 7.2 | | | |
| <i>Paracomesoma</i> | 8.36 | 21.87 | 2 | −21.2 | 4 | −19.4 ± 0.9 | 2 | 10.7 | 3 | | 8.9 ± 0.8 |
| <i>Ptycholaimellus</i> | 10.97 | 1.48 | | | 1 | −15.0 | | | | | |
| <i>Sabatieria</i> sp. 1 | 4.04 | 3.03 | 2 | −20.6 | 4 | −18.8 ± 1.2 | 1 | 7.8 | | | |
| <i>Sabatieria</i> sp. 2 | 4.04 | 3.03 | 1 | −23.4 | 1 | −28.3 | | | | | |
| <i>Sphaerolaimus</i> | 2.71 | 4.89 | 5 | −19.5 ± 1.4 | 4 | −18.7 ± 1.3 | 4 | 10.1 ± 0.1 | 3 | | 9.4 ± 0.3 |
| <i>Spirinia parasitifera</i> | 10.17 | 5.15 | 3 | −18.6 ± 3.4 | 3 | −23.3 ± 1.6 | 2 | 4.6 | 2 | | 4.8 |
| <i>Spirinia</i> sp. 2 | 10.17 | 5.15 | 2 | −15.6 | 1 | −15.9 | 1 | 7.1 | 1 | | 7 |
| <i>Terschellingia</i> | 18.13 | 25.33 | 4 | −40.9 ± 3.39 | 4 | −42.4 ± 1.1 | 2 | 3.5 | 3 | | 2.3 ± 2.5 |
| <i>Theristus</i> | 0.01 | 0.01 | 1 | −12.0 | | | | | | | |
| <i>Viscosia</i> | 0.86 | 0.80 | 1 | −18.8 | | | | | | | |
| Bulk Nematoda | | | 2 | −20.8 | 2 | −21.0 | 2 | 8.9 | 2 | | 5.6 |
| <i>COPEPODA</i> | | | | | | | | | | | |
| Cletodidae | | | 4 | −30.9 ± 3.3 | 4 | −35.5 ± 6.8 | 1 | 4.7 | | | |
| Ectinosomatidae | | | | | 1 | −18.1 | | | | | |
| <i>Sunaristes</i> (Canuellidae) | | | 1 | −19.8 | 4 | −19.2 ± 1.6 | | | 1 | | 3.9 |
| Bulk Copepoda | | | 5 | −21.7 ± 1.7 | 3 | −19.2 ± 2.5 | 4 | 6.3 ± 0.8 | 2 | | 4.7 |
| Carbon resource | | | n | Mean $\delta^{13}\text{C} \pm \text{SD}$ (‰) | | | | Mean $\delta^{15}\text{N} \pm \text{SD}$ (‰) | | | |
| Seagrass fresh leaves | | | 8 | −11.4 ± 0.7 | | | | 3.7 ± 2.1 | | | |
| Seagrass roots | | | 8 | −12.9 ± 0.4 | | | | 3.2 ± 0.7 | | | |
| Seagrass detritus | | | 4 | −15.9 ± 1.1 | | | | 3.6 ± 0.4 | | | |
| Epiphytes | | | 6 | −18.8 ± 1.8 | | | | 5.2 ± 0.7 | | | |
| Microphytobenthos (MPB) | | | 11 | −19.9 ± 1.3 | | | | 7.6 ± 1.6 | | | |
| Bulk sediment organic matter (SOM) | | | | | | | | | | | |
| 0–2 cm depth layer | | | 16 | −20.1 ± 17.7 | | | | 4.7 ± 0.2 | | | |
| 2–4 cm depth layer | | | 16 | −20.9 ± 0.8 | | | | 5.2 ± 0.8 | | | |
| 4–6 cm depth layer | | | 16 | −20.8 ± 0.7 | | | | 5.5 ± 0.2 | | | |
| Suspended particulate organic matter (SPOM) | | | 17 | −24.1 ± 1.2 | | | | 5.1 ± 1.7 | | | |

taxa with intermediate stable carbon isotope signals (Table 3). Chemoautotrophic bacteria contributed to the carbon requirements of *Terschellingia* for 0.91 (0.83–0.97) and 0.93 (0.86–0.97) in seagrass beds and in bare sediments, respectively (Table 3). It also predominantly contributed to the diet of Cletodidae: for 0.55 (0.39–0.74) in seagrass beds. In the latter, however, SPOM and MPB/epiphyte contributions were also substantial. The limited available data do not allow mixing model computations for *Sabatieria* sp. 2, although its $\delta^{13}\text{C}$ data suggest at least partly chemoautotrophic carbon utilization. Nevertheless, the contribution of the latter resource to the requirements of another species of the same

genus, *Sabatieria* sp. 1, was predicted to be low 0.14 (0.02–0.32) in seagrass beds; Table 3).

4 Discussion

4.1 Resource utilization by meiobenthos inside and adjacent to *Zostera* vegetation

The stable isotope data of resources and consumers obtained in this study suggest that seagrass detritus and roots, epiphytes, MPB and SPOM all contribute in varying degrees to the carbon requirements of meiofauna. In all, the

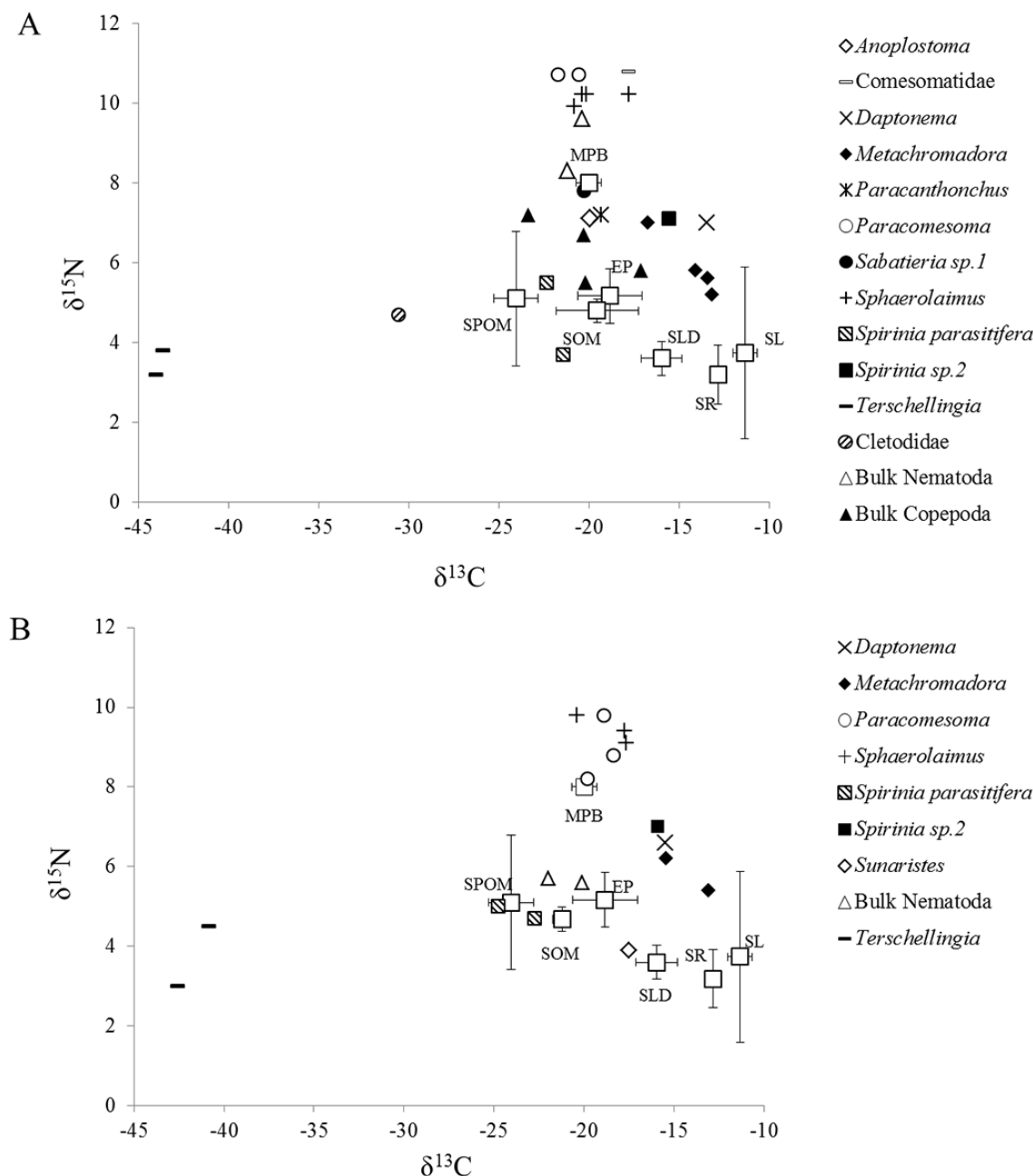


Figure 3. Biplots of $\delta^{13}\text{C} / \delta^{15}\text{N}$ of meiobenthos from the upper 2 cm and their potential resources in seagrass beds (A) and bare sediments (B). Resource data are mean values (\pm SD) of all replicate samples per source material. Abbreviations used: SL, SR and SLD for seagrass leaves, roots and detritus, respectively; EP for epiphytes, MPB for microphytobenthos, SPOM for suspended particulate organic matter and SOM for bulk sediment organic matter.

proportional contributions estimated by the isotope mixing model MixSIR agree well with our data interpretation based on the isotope biplots, despite their often wide range, given the large confidence intervals adopted for calculating the most probable model solutions. No significant differences in isotope signatures of nematode and copepod taxa inside seagrass vegetation compared to in adjacent bare

sediments were detected, contradicting our hypothesis that MPB would contribute more in bare sediments, whereas seagrass detritus and SPOM would be more important resources inside vegetated sediments. This agrees well with results for macrobenthos from the same ecosystem (Vafeiadou et al., 2013a). Seagrass vegetation has important indirect effects on resource availability, for instance, through substrate

Table 2. Mean (\pm SD) stable isotope signatures of meiofauna from the deeper sediment layers (2–6 cm), from all stations (n = number of replicate samples).

| Meiofauna | n | $\delta^{13}\text{C} \pm \text{SD} (\text{‰})$ | n | $\delta^{15}\text{N} \pm \text{SD} (\text{‰})$ |
|---------------------------------|-----|--|-----|--|
| NEMATODA | | | | |
| <i>Anoplostoma</i> | 2 | -21.6 ± 0.1 | | |
| <i>Bathylaimus</i> | 1 | -22.6 | | |
| <i>Daptonema</i> | 2 | -17.5 ± 2.3 | | |
| <i>Metachromadora</i> | 2 | -14.4 ± 0.4 | | |
| <i>Oncholaimus</i> | 2 | -26.1 ± 5.7 | | |
| <i>Paracanthochus</i> | 1 | -29.8 | | |
| <i>Paracomesoma</i> | 4 | -20.0 ± 1.5 | 2 | 7.6 ± 2.1 |
| <i>Sabatieria</i> sp. 1 | 3 | -21.1 ± 0.7 | | |
| <i>Sabatieria</i> sp. 2 | 1 | -28.6 | | |
| <i>Sphaerolaimus</i> | 1 | -23.7 | 1 | 7.5 |
| <i>Spirinia parasitifera</i> | 3 | -27.5 ± 6.2 | 1 | 4.1 |
| <i>Spirinia</i> sp. 2 | 3 | -15.9 ± 0.6 | 3 | 5.9 ± 1.1 |
| <i>Terschellingia</i> | 6 | -40.4 ± 4.5 | 1 | 3.2 |
| Bulk Nematoda | 8 | -22.3 ± 3.5 | 2 | 6.5 |
| COPEPODA | | | | |
| Cletodidae | 1 | -33.5 | | |
| Harpacticidae | 1 | -27.0 | | |
| <i>Sunaristes</i> (Canuellidae) | 1 | -15.9 | | |
| Bulk Copepoda | 7 | -22.7 ± 3.9 | | |

formation and through the enhancement of SPOM sedimentation (Ouisse et al., 2012). However, seagrass detritus and SPOM are also exported from seagrass beds to adjacent or even more distant locations (Hemminga et al., 1994; Heck et al., 2008). Our results support the idea that carbon inputs associated with seagrass beds extend beyond the vegetation boundaries and contribute to the diet of benthos living adjacent to seagrass vegetation, including representatives of the predominant meiofaunal taxa.

Fresh seagrass leaves and roots, despite their biomass, are generally considered of minor importance as carbon resources for the benthos, mainly as a consequence of their poor nutritional value and high lignocellulose content (Ott and Maurer, 1977; Vizzini et al., 2002a). This is also supported by the results of our study, where the majority of meiofaunal taxa were considerably more depleted in $\delta^{13}\text{C}$ than seagrass tissue. Nevertheless, the high contribution of seagrass carbon predicted by the mixing model and the relatively enriched $\delta^{13}\text{C}$ for some nematode genera (i.e. *Daptonema*, *Theristus*, *Metachromadora*, *Spirinia* sp. 2 and *Ptycholaimellus*) suggest that they depend to a considerable extent on seagrass-derived carbon. Based on mouth-morphology derived assumptions on their feeding ecology, these nematode genera have usually been considered MPB feeders. Our present $\delta^{13}\text{C}$ data do not point at a major contribution of MPB in the diet of these nematodes. In contrast, they clearly indicate that they utilize *Zostera* detritus, either directly or through grazing on detritivorous (micro-)organisms. In addition, exudates secreted by seagrass roots may be directly or indirectly utilized by meiofauna, for instance, through grazing on bacteria. However, Boschker et al. (2000) found no significant transfer of labelled carbon

from living seagrass tissues to benthic bacteria through root exudation. Hence, our data suggest that several abundant nematode genera utilize seagrass detritus and/or its associated micro-organisms.

The predominant aboveground associates of seagrass are epiphytic microalgae, which can contribute significantly to the primary production in seagrass beds, and have a generally high nutritional value (Kitting et al., 1984; Gambi et al., 1992; Moncreiff and Sullivan, 2001). In our study, they had considerably more depleted carbon isotope signatures than fresh or detrital seagrass material and a variety of meiofauna, in particular, several epistratum-feeding nematodes and harpacticoid copepods, had $\delta^{13}\text{C}$ values closely resembling those of epiphytes. Given the expected importance of microalgae as food to many harpacticoid copepods (De Troch et al., 2005a, b) and epistratum-feeding nematodes (Moens and Vincx, 1997), it is tempting to interpret these results as an important utilization of seagrass epiphytes by meiofauna. However, the carbon isotope signatures of epiphytes in our study overlap with those of MPB, rendering firm conclusions on the relative importance of these resources difficult (see Vafeiadou et al., 2013a). Since larger seagrass fragments were very scant on bare sediments, it is nevertheless unlikely that epiphytes would have substantially contributed to nematode diets in these bare sediments. Given the absence of significant differences in nematode isotope signatures between vegetated and bare sediments, we therefore conclude that MPB and not epiphytes is probably the most important carbon resource for these nematodes, independent of the habitat where they were collected.

Indeed, the few studies which have previously looked at resource utilization of intertidal meiofauna at genus or species level have all stressed the importance of MPB as a principal food resource (Carman and Fry, 2000; Moens et al., 2002, 2005, 2013; Rzeznik-Orignac et al., 2008; Maria et al., 2012). A number of epistratum- and deposit-feeding nematodes in our study had intermediate carbon isotope signatures, suggesting they indeed feed predominantly on MPB and/or epiphytes. However, we cannot exclude that they utilize a mix of more ^{13}C -depleted (e.g. SPOM) and more ^{13}C -enriched (e.g. seagrass detritus) food resources, which would equally result in intermediate carbon isotopic signatures.

Given the increased sedimentation in seagrass beds, and the high contribution of SPOM in intertidal areas which are characterised by higher sedimentation (Moens et al., 2005), we expected SPOM to be a comparatively more important carbon resource for meiofauna inside *Zostera* patches than in bare sediments in our study area. The carbon isotope signatures of SPOM in our study were clearly more depleted than those of the other potential resources, and in the range of “typical” values for SPOM (comparing with SPOM data from the Mondego estuary, Portugal; Baeta et al., 2009 and from the Scheldt estuary, the Netherlands; Moens et al., 2005). This was not, however, reflected in more depleted $\delta^{13}\text{C}$ signatures of meiofauna inside seagrass vegetation.

Table 3. Proportional contributions per unit of each resource to the carbon requirements of meiofauna taxa in seagrass beds and bare sediments, in the surface (2 cm) and deeper sediments (2–6 cm), as computed by the isotope mixing model MixSIR (values given as median and lower to upper limits of 95 % confidence intervals). MPB stands for microphytobenthos and SPOM for suspended particulate organic matter.

| Proportional contribution of resources | | | | |
|---|-----------------------------|-------------------|------------------|---------------------------|
| Consumers | Seagrass roots and detritus | Epiphytes and MPB | SPOM | Chemoautotrophic bacteria |
| Seagrass beds (upper 2 cm) | | | | |
| <i>Anoplostoma</i> | 0.32 (0.04–0.64) | 0.18 (0.01–0.65) | 0.26 (0.02–0.69) | 0.13 (0.02–0.31) |
| <i>Comesomatidae</i> | 0.34 (0.05–0.63) | 0.22 (0.02–0.66) | 0.24 (0.02–0.62) | 0.12 (0.01–0.28) |
| <i>Daptonema</i> | 0.70 (0.48–0.87) | 0.14 (0.01–0.42) | 0.08 (0.01–0.26) | 0.04 (0.00–0.13) |
| <i>Metachromadora</i> | 0.75 (0.60–0.88) | 0.12 (0.01–0.31) | 0.08 (0.01–0.21) | 0.03 (0.00–0.09) |
| <i>Paracanthonchus</i> | 0.36 (0.06–0.66) | 0.18 (0.01–0.66) | 0.24 (0.02–0.65) | 0.11 (0.01–0.28) |
| <i>Paracomesoma</i> | 0.25 (0.03–0.51) | 0.14 (0.01–0.48) | 0.29 (0.03–0.73) | 0.25 (0.09–0.40) |
| <i>Sabatieria</i> sp. 1 | 0.30 (0.04–0.60) | 0.19 (0.02–0.63) | 0.27 (0.02–0.68) | 0.14 (0.02–0.32) |
| <i>Sphaerolaimus</i> | 0.39 (0.07–0.59) | 0.08 (0.01–0.31) | 0.27 (0.02–0.75) | 0.22 (0.04–0.35) |
| <i>Spirinia parasitifera</i> | 0.29 (0.03–0.60) | 0.11 (0.01–0.54) | 0.37 (0.25–0.78) | 0.15 (0.02–0.33) |
| <i>Spirinia</i> sp. 2 | 0.59 (0.28–0.81) | 0.16 (0.01–0.58) | 0.12 (0.01–0.38) | 0.06 (0.01–0.13) |
| <i>Terschellingia</i> | 0.02 (0.00–0.08) | 0.02 (0.00–0.08) | 0.03 (0.00–0.10) | 0.91 (0.83–0.97) |
| <i>Cletodidae</i> | 0.12 (0.01–0.33) | 0.10 (0.01–0.33) | 0.15 (0.01–0.47) | 0.55 (0.39–0.74) |
| Seagrass beds (deeper sediments: 2–6 cm) | | | | |
| <i>Paracomesoma</i> | 0.46 (0.14–0.72) | 0.22 (0.02–0.67) | 0.17 (0.02–0.47) | 0.07 (0.01–0.22) |
| <i>Sphaerolaimus</i> | 0.20 (0.02–0.49) | 0.14 (0.01–0.51) | 0.28 (0.02–0.73) | 0.28 (0.10–0.48) |
| <i>Spirinia</i> sp. 2 | 0.72 (0.52–0.85) | 0.06 (0.01–0.27) | 0.11 (0.01–0.35) | 0.07 (0.01–0.17) |
| <i>Terschellingia</i> | 0.03 (0.00–0.11) | 0.03 (0.00–0.10) | 0.04 (0.00–0.13) | 0.89 (0.78–0.96) |
| Consumers | Seagrass roots and detritus | Epiphytes and MPB | SPOM | Chemoautotrophic bacteria |
| Bare sediments (upper 2 cm) | | | | |
| <i>Daptonema</i> | 0.71 (0.31–0.89) | 0.16 (0.01–0.60) | 0.07 (0.01–0.23) | 0.03 (0.00–0.09) |
| <i>Metachromadora</i> | 0.85 (0.70–0.95) | 0.07 (0.01–0.23) | 0.04 (0.00–0.13) | 0.01 (0.00–0.05) |
| <i>Paracomesoma</i> | 0.39 (0.06–0.69) | 0.06 (0.00–0.31) | 0.39 (0.02–0.78) | 0.12 (0.01–0.27) |
| <i>Sphaerolaimus</i> | 0.52 (0.10–0.71) | 0.07 (0.01–0.42) | 0.20 (0.01–0.72) | 0.16 (0.01–0.26) |
| <i>Spirinia parasitifera</i> | 0.34 (0.04–0.65) | 0.11 (0.01–0.53) | 0.34 (0.02–0.76) | 0.14 (0.02–0.28) |
| <i>Spirinia</i> sp. 2 | 0.67 (0.25–0.86) | 0.15 (0.01–0.63) | 0.10 (0.01–0.30) | 0.04 (0.00–0.12) |
| <i>Terschellingia</i> | 0.02 (0.00–0.06) | 0.02 (0.00–0.07) | 0.02 (0.00–0.08) | 0.93 (0.86–0.97) |
| <i>Sunaristes</i> (Canuellidae) | 0.64 (0.20–0.84) | 0.14 (0.01–0.66) | 0.11 (0.01–0.35) | 0.05 (0.00–0.14) |
| Bare sediments (deeper sediments: 2–6 cm) | | | | |
| <i>Paracomesoma</i> | 0.29 (0.03–0.64) | 0.15 (0.01–0.64) | 0.31 (0.02–0.75) | 0.14 (0.02–0.30) |
| <i>Spirinia parasitifera</i> | 0.23 (0.02–0.59) | 0.14 (0.01–0.59) | 0.34 (0.02–0.79) | 0.17 (0.03–0.35) |

Isotopic signatures reflecting utilization of SPOM were most prominent in the nematodes *Spirinia parasitifera*, *Sabatieria* sp. 2, *Oncholaimus*, *Sphaerolaimus* and *Paracomesoma*, and in the copepod family Harpacticidae from deeper sediments (2–6 cm). This was the case in both vegetated and bare sediments, except for *Oncholaimus* and Harpacticidae which occurred only in seagrass beds. The increased contributions of SPOM for the nematodes *Sphaerolaimus*, *Paracomesoma* and *Spirinia parasitifera* also confirm their reliance on this carbon resource. However, according to their elevated $\delta^{15}\text{N}$,

the first two of these genera utilize SPOM indirectly, probably through feeding on prey which rely on SPOM. Alternatively, it is also possible that the abundant genus *Terschellingia* is among their prey and therefore, indirect reliance on chemosynthetic bacteria is also possible. This is also indicated by the predicted contributions of the latter resource for these two nematode genera. In general, modelled contributions of SPOM are considerably higher when chemoautotrophic carbon is not included as a resource in the mixing model.

Furthermore, our data highlight differential resource utilization between surface (2 cm) and deeper sediment layers (2–6 cm), indicating a shift towards a higher SPOM contribution in deeper sediments for the nematodes *Anoplostoma*, *Bathylaimus*, *Oncholaimus*, *Paracanthonus*, *Sphaerolaimus* and *Spirinia parasitifera* and for the copepod family Harpacticidae. Hence, our data partly support Rudnick's (1989) hypothesis of differential resource utilization by surface-inhabiting vs. deeper-dwelling meiofauna. Surface food-addition experiments in subtidal (Ólafsson et al., 1999) and intertidal (Moens et al., 2002) sediments have also demonstrated that nematodes from both surface and deeper sediment layers can consume deposited phytodetritus. However, our results do not support Rudnick's (1989) contention that deeper-dwelling nematodes rely more on refractory organic matter. Among the resources considered in the present study, seagrass detritus is the most refractory, but our data indicate that it is utilized less rather than more by deeper-dwelling nematodes.

The strongly depleted $\delta^{13}\text{C}$ values of the nematode *Terschellingia* and the copepod family Cletodidae demonstrate utilization of a carbon resource not included in our sampling. Several chemoautotrophic processes yield highly depleted $\delta^{13}\text{C}$ values. Among them is sulfide oxidation; sulfide-oxidizing bacteria have $\delta^{13}\text{C}$ values which tend to be (well) below -30‰ (Robinson and Cavanaugh, 1995). Hence, our results strongly indicate that *Terschellingia* and Cletodidae rely predominantly or even exclusively on such bacteria, as also supported by the high contributions predicted by the mixing model.

Our data for *Terschellingia* are consistent with previous records ($\delta^{13}\text{C} = -43\text{‰}$) from a mangrove ecosystem (T. Moens, unpublished data; in Bouillon et al., 2008) and from an estuarine intertidal flat in the Oosterschelde, the Netherlands (Moodley et al., unpublished data; in Moens et al., 2011). *Terschellingia* is a microvore with a very small buccal cavity, enabling ingestion of only bacteria-sized particles, and tends to be very abundant in hypoxic/anoxic sediments (Steyaert et al., 2007), where chemosynthetic processes can be important. The nematode genera *Terschellingia* and *Sabatieria* have been suggested to feed on sulfide-oxidizing bacteria in deep-sea sediments too (Pape et al., 2011; Guilini et al., 2012). *Sabatieria* sp. 2 in our study also had depleted $\delta^{13}\text{C}$ (-23.4‰ and -28.3‰ in vegetated and bare sediments, respectively). These data suggest that *Sabatieria* sp. 2 partly relies on chemoautotrophic carbon, especially in bare sediments; in contrast, *Sabatieria* sp. 1 was more enriched than its congener and probably depends largely on MPB.

Little is known on the autecology and feeding habits of Cletodidae copepods (Hicks and Coull, 1983), but diatoms, detritus and bacteria have all been listed as their food resources (Ivester and Coull, 1977). However, recent data from a salt marsh gully in the Scheldt estuary, the Netherlands, confirm our results that sulfide-oxidizing bacteria are the ma-

jor carbon resource for these copepods (Cnudde et al., 2014). Further, Grego et al. (2014) found representatives of the family Cletodidae to be the most resistant copepods to long-term anoxia. Apart from a single mention of equally depleted $\delta^{13}\text{C}$ of an unidentified harpacticoid copepod from the Oosterschelde estuary (Moens et al., 2011), these data provide the first evidence of a trophic association between harpacticoid copepods and chemoautotrophic bacteria. Whether this association involves (selective) grazing on chemoautotrophic bacteria or some form of symbiosis remains unknown, both for the Cletodidae and for *Terschellingia*. In contrast to nematodes belonging to the Stilbonematinae (Ott et al., 1991), neither *Terschellingia* nor Cletodidae show obvious signs of ectosymbiotic micro-organisms. The possibility of an endosymbiotic relationship remains to be investigated.

4.2 Implications for nematode trophic guild classifications

A clear distinction among trophic levels within the meiofauna analysed here is evident from the stable nitrogen isotope data, with *Sphaerolaimus*, *Paracomesoma* and unidentified Comesomatidae belonging to a higher trophic level than all other nematodes and harpacticoid copepods. Our results on *Sphaerolaimus* are in agreement with trophic guild classifications based on mouth morphology (Moens and Vincx, 1997), and with results from a stable isotope study from the Scheldt estuary, the Netherlands (Moens et al., 2005) and from a mudflat in Marennes-Oléron bay, on the French Atlantic coast (Rzeznik-Orignac et al., 2008). Furthermore, predation by *Sphaerolaimus* may be selective, since its relatively depleted carbon isotope signatures poorly reflect those of the majority of its candidate prey species. On the other hand, the $\delta^{13}\text{C}$ of *Sphaerolaimus* may also result from predation on *Terschellingia* in addition to feeding on other prey species.

A predatory feeding ecology for *Paracomesoma* and unidentified Comesomatidae is, however, counter to expectations. Comesomatidae are generally considered deposit feeders (Wieser, 1953; Moens and Vincx, 1997), the prime food resources of which in intertidal and shallow subtidal sediments are often microalgae and prokaryotes (Wieser, 1953; Moens and Vincx, 1997; Moens et al., 2005). However, buccal cavities without teeth or tooth-like structures may still serve predatory strategies through ingestion of whole prey (Moens and Vincx, 1997), and a variety of ciliates and flagellates may potentially serve as first-level consumers which could be preyed upon by nematodes such as *Paracomesoma*. Similarly, Moens et al. (2005) found an unexpectedly high $\delta^{15}\text{N}$ for *Ascolaimus elongatus*; they also mentioned an unpublished observation of another comesomatid, *Sabatieria*, regurgitating ciliates upon addition of a chemical fixative. Hence, we suggest that *Paracomesoma* and unidentified Comesomatidae obtain most of their carbon through predation on heterotrophic protists or other small prey which in turn depend on various resources.

The nematode genera *Daptonema* and *Theristus* are considered non-selective deposit feeders (Wieser, 1953) or deposit feeders, which ingest suitably sized food particles like microalgae cells (Jensen, 1987; Moens and Vincx, 1997). Diatom grazing has been reported as a main feeding strategy for *Daptonema* from temperate tidal flats, based on observations (Nehring, 1990; Moens and Vincx, 1997) as well as on natural stable carbon isotope signatures (Carman and Fry, 2002; Moens et al., 2002; Rzeznik-Orignac et al., 2008). Nevertheless, the stable isotope signatures of *Spartina* sp. and MPB are often in the same range; thus, discrimination between the utilization of these two resources based on stable carbon isotopes can be difficult (see also Couch, 1989). In light of the present results, which show that *Daptonema* can utilize vascular plant detritus, caution is due when discarding vascular-plant derived detrital resources from the diet of this and other nematodes. Documentation of the feeding behaviour of intertidal *Theristus* is sparser than for *Daptonema*, but here too, diatoms have been shown to be a prominent food resource based on observations and on stable isotope data (Boucher, 1973; Moens et al., 2013). In general, however, (non-selective) deposit feeders are considered opportunistic feeders capable of ingesting a variety of food particles, including microalgae, bacteria, and perhaps also small detrital particles, the latter also being indicated by the results of this study, with particle size being a major determinant of food selection (Moens and Vincx, 1997).

A strong link between the genera *Metachromadora* and *Ptycholaimellus* and seagrass detritus was unexpected. Both genera were originally considered predators based on their mouth morphology (Wieser, 1953), but observations on feeding behaviour (Moens and Vincx, 1997) and stable isotope data (Moens et al., 2002, 2005) have shown that they can predominantly rely on MPB in intertidal flats. As epistrate feeders, they utilize a tooth to pierce food particles before emptying them, or to scrape off epigrowth from sediment or detrital particles. The present results, however, suggest that they may also utilize microbes associated with vascular plant detritus, a trophic link also suggested for *Ptycholaimellus* and *Spartina alterniflora* (Loisel.) in salt marsh sediments (Carman and Fry, 2002). Such differences between studies may point at a considerable flexibility in resource utilization (Moens et al., 2004). In any case, these results highlight that the idea that epistratum-feeding nematodes from intertidal and shallow subtidal sediments primarily utilize microalgae cannot be generalized.

Thus, we found unexpected resource utilization patterns for some deposit and epistrate feeders. In addition, we observed considerable variation in both resource use and trophic level among genera from the same feeding type (e.g. *Paracomesoma*, *Sabatieria* and unidentified Comesomatidae), showing that stoma morphology-based classifications provide very artificial functional groupings. It must be noted that all the resources considered in the present study are composed of different species (for instance for MPB/epiphytes)

or compounds (for instance different tissues and “chemical” composition in seagrass detritus), which may exhibit differences in isotopic signature. Rzeznik-Orignac et al. (2008), for instance, found small differences ($\sim 1\text{--}2\text{‰}$) in $\delta^{13}\text{C}$ between different size groups of MPB. Selective consumption of specific taxa or compounds in a resource class, or of microbes which have selectively assimilated specific compounds, may affect any interpretation of resource utilization using broadly defined resources as we have done here. Such a level of understanding would require the use of pulse-chase experiments and compound-specific rather than bulk tissue isotopic analyses (Boschker and Middelburg, 2002; De Troch et al., 2012). Nevertheless, considering the strong variation of isotope data among confamilial and even congeneric species (as observed for Comesomatidae, the two *Sabatieria* species and the two *Spirinia* species in the present study), we strongly recommend avoiding interpretation of meiofaunal resource use and even trophic level at suprageneric levels, and emphasize that resource use may be highly species-specific. Hence, we clearly demonstrate that the traditional feeding type classifications of nematodes based on buccal morphology can be misleading and should be combined with empirical information for reliable conclusions.

5 Summary

The organic carbon inputs in the benthic food web in seagrass beds at the Mira estuary derive from various resources, namely seagrass detritus, roots, epiphytes, MPB and SPOM, all to some extent being utilized by nematodes and harpacticoid copepods. In addition, chemoautotrophic carbon is also included in the diet of some taxa, most probably via feeding on sulfide-oxidizing bacteria. Seagrass detritus is available also in the bare sediments adjacent to seagrass beds, as well as in deeper layers, demonstrating the important role of seagrass-derived carbon for the estuarine benthos. The predatory feeding mode suggested for the expected deposit-feeding Comesomatidae, in addition to the considerable variation in both resource use and trophic level found for confamilial or congeneric nematode species, clearly demonstrate that the traditional feeding type classifications based on the mouth morphology of nematodes can be strongly misleading. Therefore, we recommend combining mouth morphology with stable isotope analysis at the genus or even species level in order to clarify the complex feeding interactions at/near the basis of the benthic food web.

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